

**PCT**

**NOTIFICATION OF ELECTION**

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year)

25 October 1999 (25.10.99)

International application No.

PCT/US99/01087

Applicant's or agent's file reference

160180.90121

International filing date (day/month/year)

19 January 1999 (19.01.99)

Priority date (day/month/year)

20 January 1998 (20.01.98)

Applicant

SIMS, Peter, J. et al

1. The designated Office is hereby notified of its election made:



in the demand filed with the International Preliminary Examining Authority on:

18 August 1999 (18.08.99)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was



was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Eugénia Santos

Telephone No.: (41-22) 338.83.38

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>160180.90121</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/US 99/ 01087</b>	International filing date (day/month/year) <b>19/01/1999</b>	(Earliest) Priority Date (day/month/year) <b>20/01/1998</b>
Applicant <b>BLOOD CENTER RESEARCH FOUNDATION et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☒ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☒ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ Certain claims were found unsearchable (See Box I).

3. ☒ Unity of invention is lacking (see Box II).

4. With regard to the title,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☒ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

5

☐ None of the figures.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 01087

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1,4-16,18,23-26 (as far as in vivo methods are concerned) and claims 3-17 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-11

1.1. Claims: 1-4, 6 (partially) and claim 5

A method for extending the viability of mammalian cells by inhibiting PL scramblase with an antisense RNA.

1.2. Claims: 1-4,6-7 (all partially) and claim 9

A method for extending the viability of mammalian cells by inhibiting PL scramblase with a mutant PL scramblase.

1.3. Claims: 1-4,6-7 (all partially) and claim 8

A method for extending the viability of mammalian cells by inhibiting PL scramblase with a truncated PL scramblase.

1.4. Claims: 1-4,6 (all partially) and claims 10 and 11

A method for extending the viability of mammalian cells by inhibiting or removing fatty-acid acylation of PL scramblase.

2. Claims: 12-18 and 23-26

A method for decreasing the viability, metastatic or invasive potential of cells, or for inducing apoptosis.

3. Claims: 19-22 and 27-28

A method for determining the status of a patient's cancer.



## PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT<sup>PCT</sup>

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference <b>160180.90121</b>	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. <b>PCT/US99/01087</b>	International filing date (day/month/year) <b>19/01/1999</b>	Priority date (day/month/year) <b>20/01/1998</b>
International Patent Classification (IPC) or national classification and IPC <b>C12N15/12</b>		
Applicant <b>BLOOD CENTER RESEARCH FOUNDATION et al.</b>		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 7 sheets, including this cover sheet.

- ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand  <b>18/08/1999</b>	Date of completion of this report  <b>16.05.2000</b>
Name and mailing address of the international preliminary examining authority:   <b>European Patent Office</b> <b>D-80298 Munich</b> <b>Tel. +49 89 2399 - 0 Tx: 523656 epmu d</b> <b>Fax: +49 89 2399 - 4465</b>	Authorized officer  <b>Paresce, D</b>  Telephone No. <b>+49 89 2399 8995</b> 

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US99/01087

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-72 as originally filed

**Claims, No.:**

1-28 as originally filed

**Drawings, sheets:**

1/20-20/20 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.  
☒ paid additional fees.  
☐ paid additional fees under protest.  
☐ neither restricted nor paid additional fees.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US99/01087

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
- ☐ complied with.
- ☒ not complied with for the following reasons:

**see separate sheet**

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
- ☒ all parts.
- ☐ the parts relating to claims Nos. .

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Yes: Claims 8-28
	No: Claims 1-7
Inventive step (IS)	Yes: Claims 8-28
	No: Claims
Industrial applicability (IA)	Yes: Claims 2, 19-22, 27-28
	No: Claims

2. Citations and explanations

**see separate sheet**

**Re Item I**

**Basis of the report**

- 1) The documents mentioned in this communication are numbered as in the search report, i.e. D1 corresponds to the first document of the search report.
- 2) The International Searching Authority (ISA) found multiple inventions in the present international application. The IPEA agrees with ISA's opinion as to a lack of unity of the claimed subject-matter.

According to Rule 13.1 PCT, the international application shall relate to one invention or a group of inventions so linked as to form a single general new and inventive concept. The link between the claims must be a technical relationship which finds expression in the claims themselves and the link must unify the invention to form a single general inventive concept. In our view, the claims relate to three different inventions. The separate inventions/groups of invention are:

- 1) Claims 1-11, are directed to methods for **extending** the viability of mammalian cells or tissues by inhibiting PL scramblase.
- 2) Claims 12-18 and 23-26 are directed to a method for **decreasing** the viability, metastatic or invasive potential of cells or for inducing apoptosis by causing increased expression or activity of PL scramblase.
- 3) Claims 19-22 and 27-28, are directed to a method for determining the status of a patient's cancer.

The separate inventions/groups of invention are not so linked as to form a single general new and inventive concept (Rule 13.1 PCT) for the reasons given by the ISA (please see Form PCT/ISA/206, p.1-2).

In particular, the IPEA considers that, due to the fact that the human plasma membrane phospholipid scramblase is known from the prior art and has been cloned and sequenced (D6), and its function as a mediator of transbilayer movement of plasma membrane phospholipids is known (D6) and that methods of use of inhibitors of PL scramblase, such as monoclonal antibodies against the PL

scramblase or antisense DNA derived from the DNA sequence encoding the PL scramblase, for prolonging the survival of circulating blood cells, have already been disclosed in the prior art, (see D1, claim 32 and p. 15-16), and due to the essential differences of the identified problems and corresponding proposed solutions, and due to the fact that no other technical features can be distinguished which, in light of the prior art, could be regarded as special technical features, the IPEA considers that there is no single common inventive concept underlying the plurality of claimed inventions of the present application in the sense of Rule 13.1 PCT.

In reply to the non-unity objection raised by the IPEA in Form PCT/IPEA/405, the Applicant has paid the additional fees specified in the invitation. This written opinion, therefore, will be based on the examination of the three groups of inventions listed above.

**Re Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

Claims 1, 3-18, 23-26 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

For the assessment of the present claims 1, 3-18, 23-26 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

**Re Item V**

**Reasoned statement under Article 35(2) with regard to novelty, inventive step or**

**industrial applicability; citations and explanations supporting such statement**

**1) Novelty: Article 33(2) PCT**

The subject-matter of claims 1-7 is not considered new in the sense of Article 33(2) PCT for the following reasons:

D1 describes the role of cell surface phosphatidylserine in coagulation, programmed cell death and clearance by the reticuloendothelial system (p.1-6) and the role of PL scramblase in mediating the  $\text{Ca}^{2+}$ -activated bi-directional transport of phospholipids between the two leaflets of a lipid bilayer membrane that occurs in response to cell activation, apoptosis or cell injury (see p. 6-14). D1 discloses a phospholipid scramblase protein and its use for various therapeutic methods. In particular, D1 describes a method for prolonging survival of transplanted organs comprising the step of adding an inhibitor of PL scramblase to an organ perfusate (see D1, p. 16 or claim 30), and a method for prolonging the *in vivo* survival of circulating blood cells by exposing the blood cells to an inhibitor of PL scramblase (p. 16 or claim 32) or a method for preventing the surface exposure of plasma membrane phosphatidylserine, phosphatidylethanolamine and cardiolipin on the surface of *in vitro* stored platelets or red blood cells comprising the step of adding an inhibitor of PL scramblase (claim 23). D1 also describes several inhibitors of PL scramblase such as monoclonal antibodies against the PL scramblase, antisense DNA derived from the DNA sequence encoding the PL scramblase or a peptide derived from PL scramblase (see claims and p. 15-16).

Therefore, the IPEA considers the methods described in D1 to fall under the scope of claims 1-7 of the present application.

In general, D1 relates to the idea that PL scramblase is normally required for effective clot formation and for efficient hemostasis whereas inhibition of PL scramblase activity leads to retarded blood clotting. D1, therefore, describes methods to selectively activate PL scramblase function for the acceleration of hemostasis and in preventing blood loss and methods to selectively inhibit PL scramblase function to treat thrombotic disorders characterized by excessive clot

formation (see p. 14).

The present application, on the other hand, relates to the relationship of PL scramblase function to programmed cell death and/or cancer. The present application relates to the discovery that the level of expression of PL scramblase influences the capacity of cells to resist complement injury or to succumb to stimuli that induce programmed cell death (p.14). The technical features given in claims 1-7, however, are not sufficient to allow one to distinguish the claimed methods from those described in D1. The subject-matter of claims 1-7 should be more clearly differentiated from the methods of the prior art to be considered novel over D1.

The methods described in claims 8-11 for extending the viability of mammalian cells or tissues by inhibiting PL scramblase, methods for decreasing the viability, metastatic or invasive potential of cells or for inducing apoptosis by causing increased expression or activity of PL scramblase and methods for determining the status of a patient's cancer described in claims 19-22 and 27-28, have not been made available to the public by any of the available prior art documents and can therefore be regarded as novel.

**2) Inventive Step: Article 33(3) PCT**

The subject-matter of claims 8-28 cannot be derived from the available prior art in an obvious manner and therefore complies with the requirements of Article 33(3) PCT.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/12, C07K 14/47, C12N 15/11, A61K 38/17, 31/70, C12Q 1/68, G01N 33/68, A61K 48/00</b>		<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 99/36536</b> <b>(43) International Publication Date:</b> 22 July 1999 (22.07.99)																																																																																																																																																																				
<b>(21) International Application Number:</b> PCT/US99/01087 <b>(22) International Filing Date:</b> 19 January 1999 (19.01.99) <b>(30) Priority Data:</b> 60/071,950 20 January 1998 (20.01.98) US <b>(71) Applicant (for all designated States except US):</b> BLOOD CENTER RESEARCH FOUNDATION [US/US]; 638 North 18th Street, P.O. Box 2178, Milwaukee, WI 53233 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SIMS, Peter, J. [US/US]; 13386 Caminito Mar Villa, Del Mar, CA 92014 (US). WIEDMER, Therese [US/US]; 13386 Caminito Mar Villa, Del Mar, CA 92014 (US). ZHAO, Ji [US/US]; Apartment 105, 309 North Solano Hills Drive, Solano, CA 92075 (US). <b>(74) Agent:</b> BAKER, Jean, C.; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202-4497 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> Without international search report and to be republished upon receipt of that report.																																																																																																																																																																					
<b>(54) Title:</b> METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM																																																																																																																																																																							
<b>(57) Abstract</b> <p>A method for extending the viability of mammalian cells or tissues comprising the step of inhibiting the expression of native PL scramblase within the cell or tissue is disclosed. In another embodiment of the invention, a method of decreasing the viability, metastatic or invasive potential of cancer cells, cancerous tissue, or viral-infected cell by causing increased expression or activity of PL scramblase protein within the cell or tissue is disclosed.</p>																																																																																																																																																																							
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METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY  
TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO  
CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority from U.S. Serial  
No. 60/071,950, filed January 20, 1998.

5 STATEMENT REGARDING FEDERALLY SPONSORED  
RESEARCH OR DEVELOPMENT

United States Government may have commercial rights  
under Grant R01 HL36946 from Heart, Lung, & Blood  
Institute, National Institutes of Health.

BACKGROUND OF THE INVENTION

10 As described in U.S. Serial No. 08/790,186, cell  
surface PS has a role in coagulation, programmed cell  
death and clearance by the reticuloendothelial system.  
U.S. Serial No. 08/790,186 also describes regulation of  
the transmembrane distribution of PS, the role of calcium  
15 in the collapse of phospholipid asymmetry, and the role  
PL translocation in Scott Syndrome.

Programmed Cell Death (Apoptosis)

Programmed cell death or apoptosis is an important  
process that is now recognized to be central to the  
20 selective elimination of mammalian cells during  
embryogenesis, tissue remodeling, the clonal selection of  
immune cells, metamorphosis and hormone-dependent  
atrophy, as well as cell death induced by chemicals or  
irradiation (reviewed by G.M. Cohen, Biochem. J. 326:1-  
25 16, 1997; D.L. Vaux and A Strasser, Proc. Natl. Acad.

Sci. USA 93:2239-2244; D. J. McConkey and S. Orrenius, Biochem Biophys. Res. Commun. 239:357-366, 1997, and references therein). Inappropriate apoptosis (either unregulated excessive or inappropriately low rate) is now

5 implicated in many human diseases, including Alzheimer's and Huntington's disease, autoimmune disorders, ischemic damage, and several forms of cancer ( M.E. Peter, et al., Proc. Natl. Acad. Sci. USA 94:12736-12737, 1997).

Although the specific pathways leading to cell death may

10 vary for different cell types and cell stimuli, it is convenient to divide the process into distinct phases. The earliest phase is the stimulus that initiates the apoptotic response. This may be an external signal delivered through surface receptors such as CD95 (Fas) or

15 tumor necrosis factor receptor, or may originate inside the cell in response to a drug, irradiation or toxin. In the next phase, the signal is detected and transduced to the cell death effector machinery. The third phase of the apoptotic process includes activation of proteases

20 (including caspases), as well as their positive and negative regulators (including Bcl-2, Bcl-x<sub>L</sub>, and their inhibitors Bax, Bad, and Bak) (J.C. Reed, Nature 387:773-776, 1997; T. Patel, et al., FASEB J. 10:587-597, 1996; G. M. Cohen, supra, 1997). Activation of the "execution

25 phase" results in morphological changes including cell shrinkage, cell surface exposure of aminophospholipids resulting in the recognition and phagocytosis of apoptotic cells, and plasma membrane blebbing. In the

last phase, chromatin condensation and degradation of DNA is observed. Internucleosomal degradation of DNA into multiples of 180-200 base pair fragments, recognized as "DNA laddering", has long been considered a biochemical hallmark of apoptosis, although more recent work has shown that the formation of larger DNA fragments, most notably of 50 and 300 kilobases, precedes oligonucleosomal DNA fragmentation in all systems investigated (A.H. Wyllie, Nature 284:555-556, 1980; D.G. Brown, et al., J. Biol. Chem. 268:3037-3039, 1993).

Phosphatidylserine Exposure in Apoptosis

In recent years it has become clear that externalization of plasma membrane phosphatidylserine (PS) is universally observed in cells undergoing apoptosis, although the signal transduction pathways leading to surface exposure of PS are poorly understood (B. Verhoven, et al., J. Exp. Med. 182:1597-1601, 1995; S.J. Martin, et al., J. Exp. Med. 182:1545-1556, 1995; D.L. Bratton, et al., J. Biol. Chem. 272:26159-26165, 1997.) PS exposure has been reported to be inhibited by the caspase inhibitor zVAD, implicating this family of "executioner" proteases in this process (S.J. Martin, et al., J. Biol. Chem. 271:28753-28756, 1996). In some cells, inhibitors of calpain have also been reported to inhibit externalization of PS. However, the molecular link between protease activation and PS exposure remains to be elucidated. As disclosed in US Patent Application Serial No. 08/949,246 and reported in Zhou, et al. (Q.

Zhou, et al., J. Biol. Chem. 272(29):18240-18244, 1997), we recently cloned a protein, PL scramblase, that mediates the  $\text{Ca}^{2+}$ -activated bi-directional transport of phospholipids between the two leaflets of a lipid bilayer membrane. This protein has been shown to transport phospholipids independent of the lipid headgroup, and we now have evidence that PL scramblase is mediating surface exposure of plasma membrane PS in all cells undergoing apoptosis (see below).

#### 10 Role of Ceramide in Apoptosis

In recent years data have accumulated that suggest a major role for the sphingolipid ceramide as a signal transducer in the apoptotic process. It is now evident that in response to a number of stimuli, including tumor necrosis factor, ligand binding or cross-linking of Fas, serum starvation, or  $\gamma$ -irradiation, production of ceramide is observed (Y. Hannun, Nature 274:1855-1859, 1996; R. Kolesnick and Z. Fuks, J. Exp. Med. 181:1949-1952, 1995). Conversely, exogenous addition of ceramide to cells induces apoptosis. Activation of the so-called sphingomyelin cycle results in activation of sphingomyelinase which hydrolyses plasma membrane sphingomyelin to generate cellular ceramide. Ceramide, in turn, has emerged as a candidate intracellular mediator, affecting the activities of several enzymes, including a ceramide-activated protein phosphatase (CAPP), ceramide-activated kinases (CAPK, JNK/SAPKs), and protein kinase C (I. Herr, et al., EMBO J. 16:6200-6208,

1997). In addition to activation of sphingomyelinase, *de novo* synthesis of ceramide in response to an apoptotic stimulus has also been considered. The signal transduction pathways leading to activation of sphingomyelinase remain largely unresolved. It has been noted that cytokine response modifier A (CrmA), a potent inhibitor of caspases, inhibited ceramide formation and tumor necrosis factor-induced cell death, suggesting a role for proteases in the signaling and/or activation phase of apoptosis upstream of ceramide formation (G. Ddaibo, *et al.*, *J. Exp. Med.* 185:481-490, 1997). As sphingomyelin is normally located primarily in the outer leaflet of the plasma membrane (A.J. Schroit and R.F.A. Zwaal, *supra*, 1991), it is likely that ceramide generation in the resting cell is limited by the restricted accessibility of sphingomyelin for hydrolysis by the cytoplasmic enzyme, sphingomyelinase, and that the plasma membrane phospholipid rearrangement upon induction of apoptosis results not only in surface exposure of PS, but also movement of sphingomyelin from outer to the inner leaflet of the plasma membrane. As recent evidence from our laboratory suggests that activation PL scramblase is involved in the externalization of PS in apoptotic cells and in the accelerated inward movement of the polar phospholipids phosphatidylcholine and sphingomyelin, we propose that activation of this pathway also provides additional sphingomyelin to the inner cytoplasmic leaflet of the plasma membrane as substrate

for ceramide production. As detailed below, this invention relates to compositions and methods for controlling cell and tissue susceptibility to apoptosis by modifying the transmembrane movement of the plasma membrane phospholipids between inner and outer leaflets.

#### Role of Aminophospholipids in Cell Clearance

In addition to the role that accelerated transbilayer movement of plasma membrane phospholipids may play in ceramide production and in transducing the apoptotic response, the concomitant *de novo* surface exposure of aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) is implicated in the activation of both complement and coagulation systems after tissue injury, and in removal of injured or apoptotic cells by the reticuloendothelial system (A.J. Schroit and R.F.A. Zwaal, Biochim Biophys. Acta 1971:313-329, 1991; V.A. Fadok, et al., J. Immunol. 148:2207-2216, 1996; R.H. Wang, et al., J. Clin. Invest. 92:1326-1335, 1993; D. Pradhan, et al., Mol. Biol. Cell 8:767-778, 1997). Although migration of these phospholipids (PL) from inner to outer plasma membrane leaflets is known to occur as an early event either in programmed cell death or in response to complement-mediated cell injury, and to be related to an elevation of intracellular  $[Ca^{2+}]$  ( $[Ca^{2+}]_c$ ), until recently, little was known about the cellular constituents that participate in this process of reorganizing the topology of the plasma membrane phospholipids.

Relationship of Plasma Membrane Phospholipids to Cancer

Transformed cancer cells exhibit the capacity to proliferate through unregulated mitotic division and to resist the normal cycle of senescence and programmed cell death characteristic of most normal untransformed cells. Additionally, malignant cancer cells *in vivo* exhibit the capacity to evade detection or injury by the body's immune defenses, including cellular killing by cytotoxic T-lymphocytes, humoral killing by antibody and complement, and removal by macrophages and other phagocytic cells of the reticuloendothelial system. Whereas there is now little information as to the specific role played by the plasma membrane phospholipids in the oncogenic and malignant potential of a cell, the potential importance of the transbilayer transport of phospholipid between leaflets of the plasma membrane to the growth behavior and senescence of a cell is suggested by evidence that (1) sphingomyelin-derived ceramide participates in the intracellular signaling that results in apoptosis (*supra*) (2) early events in both apoptosis and immune injury include the cell surface exposure of the aminophospholipids PS and PE, (*supra*) and (3) cell surface PE has been shown to promote activation of the complement system including generation of the opsin C3b and the cytolytic complex, C5b-9, whereas cell surface PS has been shown to directly promote clearance through the reticuloendothelial system, by promoting cytoadhesion and phagocytosis by tissue macrophages. (R.H. Wang, *et al.*,



J. Clin. Invest. 92:1326-1335, 1993; V.A. Fadok, et al.,  
J. Immunol. 148:2207-2216, 1992; R.F.A. Zwaal and A.J.  
Schroit, supra, 1997.) This implies that conditions that  
induce collapse of the normal plasma membrane  
5 phospholipid asymmetry, i.e., that promote the ingress of  
sphingomyelin from outer to inner plasma membrane leaflet  
and the egress of PS and PE from inner to outer leaflet,  
would serve to accelerate apoptosis (through generation  
of ceramide) and to accelerate cell removal through the  
10 reticuloendothelial system (through combined effects of  
cell surface PS and PE). Consistent with this premise,  
Der, et al. (Der, et al., Proc. Natl. Acad. Sci. USA  
95:15623-15628, 1998) reported that treatment with  
interferons markedly elevates expression of certain  
15 apoptosis-related genes, including PL scramblase. Der,  
et al. propose that the anti-tumor activity of  
interferons *in vivo* may relate to increased expression of  
PL scramblase in tumor cells containing interferon  
receptors, thereby promoting movement of PS to the cell  
20 surface and accelerating clearance through the  
reticuloendothelial system.

On the other hand, conditions that restrict or  
prevent this transbilayer movement of plasma membrane  
phospholipids would protect from cell death and promote  
25 the perpetual survival of the cell *in vivo*.

Identity of a protein mediating bidirectional transbilayer movement of plasma membrane phospholipids (PL Scramblase)

Bassé, et al. and Stout, et al. recently reported  
5 the purification and preliminary characterization of an  
integral RBC membrane protein that, when reconstituted in  
liposomes, mediates a  $\text{Ca}^{2+}$ -dependent transbilayer movement  
of PL mimicking plasma membrane PL reorganization evoked  
upon elevation of  $[\text{Ca}^{2+}]_c$  (F. Bassé, et al., J. Biol.  
10 Chem. 271:17205-17210, 1996; J.G. Stout, et al., J. Clin.  
Invest. 99:2232-2238, 1997). Evidence that a protein of  
similar function must also be present in platelets was  
recently reported by Comfurius, et al. (P. Comfurius, et  
al., Biochemistry 35:7631-7634, 1996).

15 The molecular identity of this PL scramblase protein  
including cDNA and deduced protein sequence of the full-  
length polypeptide was disclosed in US Patent Application  
Serial No. 08/949,246 and reported in Zhou, et al. (Q.  
Zhou, et al., J. Biol. Chem. 272(29):18240-18244, 1997).  
20 This report also provides evidence that this same protein  
mediates plasma membrane PL scramblase function in a wide  
variety of human cells and tissues. Furthermore, this  
report discloses cell-to-cell differences in the level of  
expression of mRNA encoding PL scramblase, implying  
25 tissue-specific regulation of gene expression.

Analysis of the cDNA-derived protein sequence  
revealed a strongly-preferred ( $p < 0.01$ ) inside-to-outside  
orientation of the predicted 19 residue transmembrane  
helix, consistent with a type II plasma membrane protein.

Most of the polypeptide (residues 1-290) thereby extends from the cytoplasmic membrane leaflet, leaving a short exoplasmic tail (residues 310-318) (Q. Zhou, et al., supra, 1997). The predicted orientation of this protein

5 is consistent with the anticipated topology of PL scramblase in the erythrocyte membrane, where lipid-mobilizing function is responsive to  $[Ca^{2+}]$  only at the endofacial surface of the membrane (Q. Zhou, et al., supra, 1997; P. Williamson, et al., Biochemistry 31:6355-

10 6360, 1992; E.F. Smeets, et al., Biochim. Biophys. Acta BioMembr. 1195:281-286, 1994; F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997; P. Williamson, et al., Biochemistry 34:10448-10455, 1995; D.L. Bratton, J. Biol. Chem. 269:22517-22523, 1994).

15 Consistent with the divalent ion-dependence of its PL-mobilizing activity, Zhou, et al. identified a conserved EF-hand-like motif in PL scramblase that is required for the  $Ca^{2+}$ -accelerated transbilayer movement of membrane phospholipids (Zhou, et al., Biochemistry

20 37:2356-2360, 1998), and PL scramblase was shown to undergo a prominent conformational change upon binding  $Ca^{2+}$  (Stout, et al., Biochemistry 37:14860-14866, 1998). Human PL scramblase was shown to be thiol-esterified with palmitate at cytoplasmic cysteinyl residue(s) and

25 evidence provided that the normal PL-mobilizing activity of this protein is dependent upon such post-translational modification of the polypeptide with fatty acid (Zhao, et al., Biochemistry 37:6361-6366, 1998).

In addition to platelet and red blood cell, PL scramblase activity has been observed in many other cells, and this  $\text{Ca}^{2+}$ -induced response is thought to be central to the rapid movement of PS and

5 phosphatidylethanolamine from inner plasma membrane leaflet to the surface of perturbed endothelium, and a variety of injured and apoptotic cells (R.F.A. Zwaal and A.J. Schroit, supra, 1997). The resulting exposure of PS at the cell surface is thought to play a key role in

10 removal of such cells by the reticuloendothelial system, in addition to activation of both the plasma complement and coagulation systems (R.H. Wang, et al., supra, 1993; V.A. Fadok, et al., supra, 1992; R.F.A. Zwaal and A.J. Schroit, supra, 1997; D. Pradhan, et al., supra, 1997).

15 Whereas the molecular mechanism(s) in each circumstance remains unresolved, evidence for a specific platelet membrane protein functioning to accelerate migration of PL between membrane leaflets at increased cytosolic  $[\text{Ca}^{2+}]$  has been reported (P. Comfurius, et al., supra, 1996;

20 Zhou, et al., supra, 1997), similar to the proposed role of PL scramblase in red blood cells (F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997). It was thus of interest to determine whether mRNA for this protein is expressed in nucleated cells where PL

25 scramblase-like activity has been observed.

Northern blotting with PL scramblase cDNA revealed transcripts of ~1.6 and ~2.6 kb in all tissues and cell lines tested (Q. Zhou, et al., supra, 1997). Some

tissue-to-tissue and cell line variability in the relative abundance of these two transcripts is apparent, the significance of which remains to be determined. Also notable was markedly reduced expression in HL-60 and the lymphoma lines Raji and MOLT-4 whereas abundant message was detected in spleen, thymus, and peripheral leukocytes. In addition to the transformed cell lines shown, mRNA for PL scramblase was also confirmed in human umbilical vein endothelial cells. Whereas these data imply that the same protein identified as mediating accelerated transbilayer flip-flop of the erythrocyte membrane PL also plays a similar role in the plasma membrane of platelets, leukocytes and other cells, actual confirmation for this role of PL scramblase awaits analysis of a cell line that is selectively deficient in this protein (Q. Zhou, et al., supra, 1997). In Scott syndrome, a bleeding disorder related to an inherited deficiency of plasma membrane PL scramblase function, erythrocytes deficient in PL scramblase activity were found to contain normal amounts of the PL scramblase protein (J.G. Stout, et al., supra, 1997). Furthermore, despite the apparent deficiency in Scott syndrome cells of endogenous PL scramblase function, when PL scramblase protein from these cells was purified and reconstituted in proteoliposomes containing exogenous PL, it exhibited normal  $\text{Ca}^{2+}$ -dependent PL-mobilizing activity (J.G. Stout, et al., supra, 1997). Consistent with these results, analysis of B lymphoblasts from a patient with Scott

syndrome by Northern blot and RT-PCR revealed normal amounts of RNA and cDNA of PL scramblase, respectively, and cDNA sequence was identical to that previously reported for PL scramblase (Q. Zhou et al., Blood 5 92:1707-1712, 1998). These results suggest that in addition to the known regulation by intracellular  $[Ca^{2+}]$ , the activity of PL scramblase in the plasma membrane is regulated by other as yet unidentified membrane or cytoplasmic component(s).

10 Relationship of PL Scramblase to Cancer

Kasukabe et al., 1997 (T. Kasukabe, et al., Blood 89:2975-2985, 1997) disclose a gene designated NOR1 that is down-regulated in transformed murine monocytes relative to amount of mRNA expressed in normal mouse 15 cells and a 5'-truncated form of this same transcript (designated TRA1) that is over-expressed in leukemogenic mouse monocytic cell lines but not found in the non-leukemogenic monocytic cell lines. They suggest that the Tra1 gene product is associated with the leukemogenesis 20 of monocytic Mm cell lines.

Analysis of the open reading frame predicted by the NOR1 and TRA1 cDNA sequences reveals near-identity of protein sequence with mouse or human PL scramblase in the overlapping portions of each polypeptide. The apparent 25 identity of the full-length NOR1 gene product as the mouse homologue of human PL scramblase was recently confirmed on the basis of cDNA cloning and sequence analysis (T. Kasukabe, et al., Biochem. Biophys. Res.

Commun. 249:449-55, 1998.) As will be detailed below, the present invention relates to our discovery that the level of expression of PL scramblase influences the capacity of cells to resist complement injury or to succumb to stimuli that induce programmed cell death. The present invention also relates to the role of PL scramblase in promoting clearance of injured or apoptotic cells by the phagocytic cells of the reticuloendothelial system.

10           Needed in the art is a method for modulating the activity or level of phospholipid scramblase within a cell, organ or tissue in which one wishes either to reduce the potential for cell death and resulting clearance of beneficial cells by the scavenger  
15           macrophages of the reticuloendothelial system (by decreasing cellular PL scramblase expression or activity) or to promote cell death and cell clearance from the body of undesirable cells (by increasing cellular PL  
20           scramblase expression or activity).

20           BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

          Fig. 1A is a comparison of the cDNA and deduced amino acid sequence of human PL scramblase (SEQ ID NOs:1 and 2).

          Fig. 1B is a comparison of the cDNA and deduced  
25           amino acid sequence of murine PL scramblase (SEQ ID NOs:3 and 4).

Fig. 2 is a bar graph illustrating immunoprecipitation of erythrocyte PL scramblase.

Fig. 3 is a graph of an activity assay of recombinant PL scramblase.

5 Fig. 4 is an immunoblot of PL scramblase in human erythrocytes and platelets.

Fig. 5 is a comparison of protein sequences of mouse and human PL scramblase (SEQ ID NOS:2 and 4).

10 Fig. 6 is a bar graph of PL scramblase activity as a function of mutational analysis of a putative EF hand loop motif contained in human PL scramblase.

Fig. 7 graphs the  $\text{Ca}^{2+}$  dependence of mutant human PL scramblase with amino acid substitutions in putative  $\text{Ca}^{2+}$  binding site.

15 Fig. 8 is a Western blot analysis of PL scramblase protein and corresponding functional assay of PL scramblase activity in various human cell lines.

Fig. 9A and B are fluorescence micrographs of GFP-PL scramblase in transformed Raji cells. Fig. 9A depicts  
20 fluorescence of cells expressing GFP; Fig. 9B depicts cells transfected with pEGFP-C2-PL scramblase plasmid and expressing GFP-PL scramblase fusion protein.

Fig. 10 is a graph showing that the level of expression of PL scramblase determines plasma membrane  
25 sensitivity to intracellular calcium.

Fig. 11 is a bar graph illustrating inactivation of PL scramblase by hydroxylamine.



Fig. 12 is analysis of metabolically-labeled PL scramblase by SDS-PAGE revealing covalent incorporation of thioester-linked fatty acid.

Fig. 13 is analysis by thin layer chromatography demonstrating that PL scramblase contains covalent palmitic acid.

Fig. 14A and B is a bar graph illustrating the role of PL scramblase in PS exposure during apoptosis of B-lymphocytes. Fig. 14A compares Raji and W9 cell lines. Fig. 14B compares individual Raji cell clones that were stably transfected with either pEGFP-C2 or with pEGFP-PL scramblase and then induced to apoptosis.

Fig. 15 is a bar graph illustrating the percent apoptotic cells for Raji cells, Raji cells transfected with GFP-PL scramblase fusion protein or GFP vector as a control.

#### DESCRIPTION OF THE INVENTION

The present invention relates to the creation and use of reagents and methods to either inhibit or promote cell injury, cell death, and cell clearance from the body. These reagents and methods rely on the properties of phospholipid scramblase (PL scramblase), a protein that mediates  $\text{Ca}^{2+}$ -dependent transbilayer movement of plasma membrane phospholipids that are involved in the activation of the plasma complement and coagulation cascades and are substrates for cellular enzyme cascades that mediate programmed cell death.

In one embodiment, the present invention is a method for decreasing the viability of cancer cells or virus-infected cells comprising the step of increasing the amount or activity of PL scramblase in the plasma membrane of the cancer cell, cancerous tissue, or virus-infected cell, thereby promoting the movement of plasma membrane phospholipids between inner and outer plasma membrane leaflets. Preferably, the increase in PL scramblase is accomplished by activating the promoter of the endogenous PL scramblase gene or by delivering cDNA encoding PL scramblase, provided in a suitable targeting vector to the cell or tissue. In a preferred embodiment, cDNA encoding PL scramblase is delivered to the cell or tissue in a targeting vector, where the vector is a retrovirus or adenovirus vector containing the PL scramblase cDNA.

The feasibility of this strategy is exemplified by a recent report by Der, *et al.* (*supra*, 1998) that treatment of a human fibrosarcoma cell line with interferons markedly elevates expression of a number of apoptosis-related genes, including PL scramblase. Der, *et al.* hypothesize that the anti-tumor activity of interferons in vivo may relate to increased expression of PL scramblase in tumor cells containing interferon receptors, thereby promoting movement of PS to the cell surface and accelerating clearance through the reticuloendothelial system.

In another embodiment, the present invention is a method for inducing programmed cell death (apoptosis) of a cell, preferably a mammalian cell, comprising the step of increasing the level of expression of plasma membrane PL scramblase, thereby increasing the movement of plasma membrane phospholipids between membrane leaflets, resulting in the movement of the phospholipid sphingomyelin from outer membrane leaflet to inner leaflet and the increase in production of ceramide from sphingomyelin through the action of the cytoplasmic enzyme sphingomyelinase. The resulting ceramide is known to induce activation of a cascade of cellular enzymes that ultimately lead to apoptotic cell death.

In another embodiment, the present invention is a method for promoting clearance of a cell from the body by phagocytic uptake into the reticuloendothelial system comprising the step of increasing the level of expression of plasma membrane PL scramblase, thereby increasing cell surface exposure of phosphatidylserine and phosphatidylethanolamine through the movement of these aminophospholipids from inner plasma membrane leaflet to outer plasma membrane leaflet. Cell-surface-exposed phosphatidylserine is known to directly promote adhesion and phagocytosis by macrophages and other cells of the reticuloendothelial system whereas phosphatidylethanolamine promotes cell adhesion and phagocytosis by macrophages and other cells of the reticuloendothelial system by promoting complement

activation, which opsonizes the cell surface with complement C3b.

In another embodiment, the present invention is a method of promoting the viability of a mammalian cell, both in *in vitro* cell culture and *in vivo*, comprising the step of decreasing the activity of the plasma membrane PL scramblase, thereby inhibiting movement of plasma membrane phospholipids between inner and outer membrane leaflets. The decrease in plasma membrane PL scramblase is accomplished by either decreasing expression of the endogenous PL scramblase gene or by delivering to the cell an inhibitor of the PL scramblase protein. In one embodiment, the expression of PL scramblase is inhibited by delivering to the cell antisense oligonucleotides or antisense cDNA to PL scramblase RNA. In another embodiment, a defective form of the PL scramblase protein is expressed in the cell and that mutant PL scramblase inhibits the endogenous PL scramblase. In the preferred embodiment, endogenous PL scramblase is inactivated by deacylating essential fatty acids from the protein that are required for normal PL scramblase function in the plasma membrane. By decreasing the activity of plasma membrane PL scramblase, the cell is protected due to decreased hydrolysis of plasma membrane sphingomyelin to ceramide (mediated by cellular sphingomyelinase), as well as by decreased exposure of the procoagulant and complement-activating aminophospholipids

phosphatidylserine and phosphatidylethanolamine at the cell surface.

In another embodiment, the present invention is a method of preventing the phagocytic removal and clearance  
5 of a transfused or transplanted cell by preventing surface exposure of plasma membrane phosphatidylserine by delivering to the cell a mutant phospholipid scramblase that inhibits the endogenous PL scramblase. This scramblase is preferably mutated at a site of post-  
10 translational modification, most preferably the site is selected from the group consisting of Asp<sup>273</sup>-Asp<sup>284</sup>, Thr<sup>161</sup> and conserved cysteines of human PL scramblase or the corresponding conserved residues in mouse or other mammalian PL scramblase.

15 In one embodiment, a gene construct encoding a mutant phospholipid scramblase is delivered to the cell. In an alternative embodiment, the mutant protein itself is delivered.

In another embodiment the present invention is a  
20 method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue. This method may comprise the step of analyzing the amount of PL scramblase RNA or protein within a patient's cell sample. We predict that the presence of a comparatively  
25 low amount of PL scramblase RNA or protein will indicate the presence of cancerous tissue. The level of PL scramblase RNA may be measured by *in situ* hybridization or Northern blotting with PL scramblase cDNA or by

polymerase chain reaction using oligonucleotide primers based on sequence of human PL scramblase RNA.

Alternatively, the method may comprise the step of analyzing the sequence of a patient's PL scramblase gene,  
5 RNA or protein for the presence or absence of mutations. This analysis may preferably be selected from the group consisting of restriction fragment length polymorphism, sequencing by RT-PCR, Northern blotting, Western blotting, electrophoretic gels, protease digestion, and  
10 other techniques designed to analyze RNA, DNA or protein sequence.

In a preferred embodiment, the present invention involves the use of a preparation of a plasma membrane phospholipid scramblase ("PL scramblase"). It is now  
15 known that PL scramblases isolated from various species have sequence variations. By "PL scramblase" we include these variants as well as the proteins as described in U.S. Serial No. 08/790,186, Serial No. 08/949,246, Zhao, et al. (J. Biol. Chem. 272(29):18240-18244, 1997), and  
20 Kasukabe, et al., (Biochem. Biophys. Res. Comm. 249:449-55, 1998) and genes or cDNAs encoding these proteins.

Preferably, the protein is approximately 35-37 kD as measured on a 12.5% SDS polyacrylamide gel under reducing conditions. In a most preferred form of this invention,  
25 the preparation is a human or a mouse PL scramblase.

In one preferred embodiment of the present invention, the PL scramblase comprises SEQ ID NO:2, representing human PL scramblase, possibly with conservative or functionally equivalent substitutions.

5 In the most preferred embodiment of the present invention, the PL scramblase has been modified by the action of mammalian cellular enzymes to covalently incorporate phosphorus at one or more Thr, Ser, or Tyr residues or a fatty acid, preferably palmitate, at a  
10 cysteine residue.

The present invention may also use a murine cell protein, wherein the protein is a plasma membrane phospholipid scramblase, preferably wherein the protein is approximately 35 kD as measured on a 12.5%  
15 SDSpolyacrylamide gel under reducing conditions.

In another preferred embodiment of the present invention, the murine PL scramblase has been modified by the action of mammalian cellular enzymes to covalently incorporate phosphorus at one or more Thr, Ser, or Tyr  
20 residues, and a fatty acid, preferably palmitate, at a cysteine residue.

The present invention may employ an inhibitor of the PL scramblase activity of PL scramblase. This inhibitor may be an antisense nucleotide derived from the DNA  
25 sequence of PL scramblase. In another embodiment, the inhibitor is a peptide sequence that is a competitive inhibitor of PL scramblase activity. In another

embodiment, the inhibitor is an antibody, preferably a monoclonal antibody, raised against PL scramblase.

In another embodiment, the inhibitor works by modifying or inhibiting the post-translational  
5 modifications of the PL scramblase that are disclosed below in the Examples. For example, analysis of the primary PL scramblase sequence reveals a potential site of phosphorylation by protein kinase C or other cellular kinase (Thr<sup>161</sup>), potential sites for acylation by fatty  
10 acid, and a potential binding site for Ca<sup>2+</sup> ion provided by an EF-hand-like loop spanning residues Asp<sup>273</sup>-Asp<sup>284</sup>. These residues and motifs are conserved in the mouse PL scramblase.

In another embodiment, the inhibitor is a compound  
15 that prevents thioacylation of the protein.

In another embodiment, a mutant phospholipid scramblase is provided in which cysteine residues have been replaced by alanine or other non-conservative substitution.

20 The present invention is also a method for promoting the viability and circulating lifetime of *in vitro* stored leukocytes, lymphocytes, platelets, or red blood cells. This method comprises the steps of adding an inhibitor of PL scramblase activity to the stored blood cells.

25 The present invention is also a method for prolonging survival of transplanted cells, tissues, or organs, comprising the step of adding an inhibitor of PL scramblase activity to an organ perfusate or cell storage



medium during *in vitro* organ storage. The present invention is also a method for prolonging the survival of transplanted cells, tissues, and organs by genetically engineering the cells to be transplanted so as to reduce  
5 their expression of plasma membrane PL scramblase in order to reduce exposure of PS and other thrombogenic and pro-inflammatory phospholipids at the plasma membrane surface. In one embodiment of the present invention, the transplanted cells to be treated are hematopoietic stem  
10 cells to be used to repopulate the bone marrow by transfusion into a patient.

The present invention is also a method for diagnosing the survivability, metastatic, and invasive potential of a cancer cell or cancerous tissue or virus-  
15 infected cell by quantifying the level of cellular expression of PL scramblase and by detecting deletion or loss of function mutations in PL scramblase. For example, this quantitation may take the form of immunoblotting using an antibody to PL scramblase, an  
20 ELISA assay using an antibody to PL scramblase, flow cytometric analysis of the binding of monoclonal antibody reactive against the predicted extracellular domain of PL scramblase (residues Ser<sup>310</sup> Trp<sup>318</sup> of sequence disclosed in SEQ ID NO:2 or the equivalent residue in the conserved  
25 region of another PL scramblase) or using oligonucleotides derived from PL scramblase cDNA and the polymerase chain reaction. In one method, Northern blotting or *in situ* hybridization is performed using cDNA

of human PL scramblase as probe. In another method of the present invention, mutations in the PL scramblase gene locus are detected by DNA sequencing of the cDNA product of the polymerase chain reaction and by analysis of restriction length polymorphisms. In another method of the present invention, the quantitation is performed by isolating PL scramblase from tissue biopsy or a patient blood sample, measuring the amount of PL scramblase isolated and comparing the measurement with a control sample. The measurement may be by isolating PL scramblase from a patient tissue biopsy or blood sample and measuring via densitometry the amount of PL scramblase protein electrophoresed in a stained electrophoretic gel.

15

## EXAMPLES

EXAMPLE 1: Cloning of Murine PL scramblase and Identity of a Conserved Motif in Phospholipid Scramblase that is Required for Accelerated Transbilayer Movement of Membrane Phospholipids by  $\text{Ca}^{2+}$  (Zhou, et al., Biochemistry 37:2356-2360, 1998).

20

A. Summary

Accelerated transbilayer movement of plasma membrane phospholipids (PL) upon elevation of  $\text{Ca}^{2+}$  in the cytosol plays a central role in the initiation of plasma clotting and in phagocytic clearance of injured or apoptotic cells. We recently identified a human erythrocyte membrane protein that induces rapid transbilayer movement

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of PL at elevated  $\text{Ca}^{2+}$ , and presented evidence that this PL scramblase is expressed in a variety of other cells and tissues where transbilayer movement of plasma membrane PL is promoted by intracellular  $\text{Ca}^{2+}$  (Q. Zhou, et al., J. Biol. Chem. 272:18240-18244, 1997). We have now cloned murine PL scramblase for comparison to the human polypeptide (Fig. 1B): Both human and murine PL scramblase are acidic proteins ( $\text{pI}=4.9$ ) with a predicted inside-outside (type 2) transmembrane segment at the carboxyl-terminus (Fig. 5). Whereas human PL scramblase (318 AA) terminates in a short exoplasmic tail, murine PL scramblase (307 AA) terminates in the predicted membrane-inserted segment. The aligned polypeptide sequences reveal 65% overall identity, including near identity through 12 residues of an apparent  $\text{Ca}^{2+}$  binding motif (D[A/S]DNFGIQFPLD) spanning residues 273-284 (human, SEQ ID NO:2) and 271-282 (murine, SEQ ID NO:4), respectively (Fig. 5). This conserved sequence in the cytoplasmic domain of PL scramblase shows similarity to  $\text{Ca}^{2+}$ -binding loop motifs previously identified in known EF-hand structures. Recombinant murine and human PL scramblase were each expressed in *E. coli* and incorporated into proteoliposomes. Measurement of transbilayer movement of NBD-labeled PL confirmed that both proteins catalyzed  $\text{Ca}^{2+}$ -dependent PL flip/flop similar to that observed for the action of  $\text{Ca}^{2+}$  at the cytoplasmic face of plasma membranes. Mutation of residues within the putative EF hand loop of human PL scramblase resulted in loss of its

PL mobilizing function, suggesting that these residues directly participate in the  $\text{Ca}^{2+}$  induced active conformation of the polypeptide.

B. Experimental Procedures

5       Abbreviations used: PL, phospholipids(s); PC, phosphatidylcholine; PS, phosphatidylserine; NBD-PC, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-sn-glycero-3-phosphocholine; EST, expressed sequence tag; MBP, maltose binding protein;  
10      PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PCR; polymerase chain reaction.

Materials: Mouse fibroblast 5'-stretch plus cDNA library and KlenTaq polymerase were obtained from CLONTECH Laboratories. Expressed sequence Tag (EST)  
15      clone with GenBank™ accession number gb AA110551 was from American Type Culture Collection (ATCC 977052).  $\alpha$ - $^{32}\text{P}$ -dCTP was purchased from Dupont. Random Primed DNA Labeling Kit was from Boehringer Mannheim. Hybond-N Nylon membrane was from Amersham. Expression vector  
20      pMAL-C2, amylose resin and all restriction enzymes were from New England Biolabs. Wizard Kit was from Promega. Qiagen Lambda Kit was from Qiagen. Egg yolk phosphatidylcholine (PC), brain phosphatidylserine (PS) and 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-sn-glycero-3-phosphocholine (NBD-PC)  
25      were obtained from Avanti Polar Lipids. Factor Xa was from Haematologic Technologies, and Bio-Beads SM-2 were from BioRad. N-octyl- $\beta$ -D-glucopyranoside and Glu-Gly-Arg

chloromethyl ketone were from Calbiochem. Sodium dithionite (Sigma) was freshly dissolved in 1 M Tris, pH 10, at a concentration of 1 M.

5     Labeling of DNA Probe: The DNA insert of EST clone gb AA110551 was released by digestion with EcoRI and ApalI and purified by Wizard Kit. Four micrograms of purified DNA were labeled with 1 mCi of  $\alpha$ - $^{32}\text{P}$ -dCTP. The specific radioactivity of the probe was  $3.9 \times 10^8$  dpm/ $\mu\text{g}$  DNA.

10    Isolation of Mouse PL Scramblase cDNA by Plaque

Hybridization: E. coli strain Y1090r was transformed by mouse fibroblast cDNA library ( $6 \times 10^5$  pfu) and poured onto 30 plates (15 cm diameter, 20,000 pfu per plate). Plaques were lifted onto Hybond-N Nylon  
15    membranes. After denaturation, neutralization and UV-cross-linking, the membranes were first prehybridized in a solution composed of 5X Denhardt, 5X SSC, 1% SDS, and 200  $\mu\text{g/ml}$  herring sperm DNA for 3 hours at  $68^\circ\text{C}$ , and then  
20    hybridized in the same solution containing 5 ng/ml  $^{32}\text{P}$ -labeled probe for 16 hours at  $68^\circ\text{C}$ . The membranes were washed once with 2X SSC, 0.1% SDS, then three times with 0.1 X SSC, 0.1% SDS for 20 minutes at  $65^\circ\text{C}$ , and exposed to X-ray film. Secondary plaque lifts and hybridization  
25    were carried out on 8 positive plaques at a density of about 100 plaques/plate. Single positive and well isolated plaques were picked and amplified.  $\lambda$ DNA was purified with Qiagen Lambda Maxi Kit.

DNA Sequencing. DNA was sequenced on an ABI DNA Sequencer Model 373 Stretch (Applied Biosystems) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer).

5        Cloning of Mouse PL Scramblase into pMAL-C2 Expression Vector. In order to express mouse PL scramblase as a fusion protein with maltose binding protein (MBP), cDNA encoding mouse PL scramblase was cloned into pMAL-C2 expression vector. PCR was performed  
10       on a mouse scramblase clone using the primers 5'TCA GAA TTC GGA TCC ATG GAG GCT CCT CGC TCA GGA AC3' (SEQ ID NO:8) with an EcoRI site before the ATG start codon and 5'GCT TGC CTG CAG GTC GAC CTA CAC ACA GCC TTC AAA AAA CAT G3' (SEQ ID NO:9) with a SalI site after the stop codon.  
15       KlenTaq polymerase was used to ensure high fidelity amplification. The PCR product was digested with EcoRI and SalI, isolated by electrophoresis, and cloned into pMAL-C2 immediately 3' of MBP. E. coli strain TB1 was transformed, and sequence of the cDNA insert of plasmid  
20       from a single colony was confirmed.

Expression and Purification of Mouse PL Scramblase-MBP Fusion Protein: Mouse PL scramblase was expressed as fusion protein with MBP in E. coli TB1 and purified on amylose resin as previously described for human PL  
25       scramblase (Q. Zhou, et al., J. Biol. Chem. 272:18240-18244, 1997). The purified fusion protein was centrifuged at 106,000 x g for 1 hour at 4°C to remove aggregated protein.

Reconstitution and Functional Activity of PL

Scramblase: Reconstitution, removal of MBP, and functional assay of PL scramblase were performed as previously described (F. Bassé, *et al.*, J. Biol. Chem. 271:17205-17210, 1996; Q. Zhou, *et al.*, *supra*, 1997; J.G. Stout, *et al.*, J. Clin. Invest. 99:2232-2238, 1997). Routinely, 420 pmoles of protein were reconstituted with 1  $\mu$ mol of PL. To remove MBP, proteoliposomes were incubated 3 hours at room temperature with 1/40 (w/w) factor Xa. The digest was terminated by addition of 100  $\mu$ M Glu-Gly-Arg chloromethyl ketone. Proteoliposomes labeled with NBD-PC were incubated for 2 hours at 37°C in Tris buffer in the presence or absence of  $\text{CaCl}_2$ , as indicated in figure legends and diluted 25-fold in Tris buffer containing 4 mM EGTA. Initial fluorescence was recorded (SLM Aminco 8000 spectrofluorimeter; excitation at 470 nm, emission at 532 nm), 20 mM dithionite was added, and the fluorescence was continuously monitored for a total of 120 seconds. Scramblase activity was calculated according to the difference in non-quenchable fluorescence observed in presence vs absence of  $\text{CaCl}_2$ . Ionized  $[\text{Ca}^{2+}]$  was calculated using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA).

Protein Concentrations: Protein concentrations were estimated based upon optical density at 280 nm, using extinction coefficients ( $\text{M}^{-1}\text{cm}^{-1}$ ) of 39,000 (PL

scramblase), 64,500 (MBP), and 105,000 (PL scramblase-MBP fusion protein).

Mutagenesis of PL Scramblase: Human PL scramblase amino acid residues in EF-hand  $\text{Ca}^{2+}$ -binding motif at positions of Asp<sup>273</sup>, Asp<sup>275</sup>, Phe<sup>277</sup>, Ile<sup>279</sup>, Phe<sup>281</sup> and Asp<sup>284</sup> were mutated to Ala with oligonucleotide-directed mutagenesis by two rounds of PCR. PL scramblase-pMAL-C2 was selected as template, and the first round of PCR was performed with pairs of a complementary oligonucleotide primer containing the point mutation plus a primer complementary to a site near the ATG initial codon or TAG stop codon. PCR products were purified by Wizard kit. Full-length mutated PL scramblase cDNA was obtained by overlapping PCR and cloned back into pMAL-C2 vector. After confirmation of correct DNA sequence the mutants were recombinantly expressed in *E. coli* as described above and analyzed by SDS-PAGE.

### C. Results and Discussion

Isolation of cDNA of Mouse PL Scramblase. Murine EST clones in GenBank containing putative PL scramblase sequence were identified by a Blast homology search using the human PL scramblase cDNA. Among several clones exhibiting significant homology, a 403 bp Stratagene mouse kidney clone (gb accession number AA110551) with 79% nucleotide sequence identity to human PL scramblase was selected and this clone was used to probe a mouse fibroblast cDNA library. Eight positive clones were identified after two rounds of plaque hybridization. Two



of the eight clones were sequenced yielding 1354 bp and 1529 bp, respectively. Alignment revealed 1261 bp of overlapping sequence that spanned an open reading frame of 921 bp and specified a total of 1622 bp of unique cDNA  
5 sequence (SEQ ID NO:3).

SEQ ID NO:4 represents the open reading frame of the translated sequence of SEQ ID NO:3 (see Fig. 1B). The deduced mouse PL scramblase cDNA encodes a 307 residue protein with a molecular weight of 33.9 kDa and a  
10 theoretical pI = 4.9, similar to values obtained for the human protein (318 residues, 35.1 kDa; pI=4.9; ref. (Q. Zhou, et al., supra, 1997)). The overall identity of the mouse and human PL scramblase is 64.8%, with the most divergent sequence generally contained in the N-terminal  
15 portion of the polypeptide (Fig. 5). Fig. 5 depicts the alignment of protein sequences of mouse and human PL scramblase. Alignment between mouse (MUR) and human (HUM) PL scramblase was performed by FASTA program using the Smith-Waterman algorithm. (W.R. Pearson and D.J.  
20 Lipman, Proc. Natl. Acad. Sci. USA 85:2444-2448, 1988) Sequence of human PL scramblase is contained in GenBank™ accession number AF008445. Amino acid identities ( : ) or similarities ( . ) between the two sequences are indicated. Also indicated are the residues comprising a  
25 predicted inside-out transmembrane domain (MUR 289-307, HUM 291-309; double underline), and the 12 residues of the acidic loop of a putative EF-hand (MUR 271-282, HUM 273-284; single underline).

In both proteins, a single 19 residue transmembrane helix is predicted at the carboxyl terminus, exhibiting a strongly preferred inside-to-outside orientation. Whereas the mouse protein terminates immediately after this conserved transmembrane helix, the human PL scramblase contains an additional nine residues, implying that the short exoplasmic peptide in human PL scramblase is non-essential to function. Homology motifs conserved in both proteins include a potential site for protein kinase C phosphorylation (Thr<sup>159</sup> in mouse, Thr<sup>161</sup> in human) and a potential Ca<sup>2+</sup>-binding EF-hand loop motif adjacent to the transmembrane helix (residue Asp<sup>271</sup> to Asp<sup>282</sup> in mouse and residues Asp<sup>273</sup> to Asp<sup>284</sup> in human). The cytoplasmic orientation of this protein and the proximity of this putative Ca<sup>2+</sup>-binding domain to the segment of polypeptide that is inserted into the plasma membrane are consistent with the proposed activity of this protein in situ, where Ca<sup>2+</sup> acting directly at the endofacial membrane surface is known to initiate the rapid transbilayer movement of plasma membrane PL (P. Williamson, et al., Biochemistry 31:6355-6360, 1992; R.F.A. Zwaal and A.J. Schroit, Blood 89:1121-1132, 1997; F. Bassé, et al., J. Biol. Chem. 271:17205-17210, 1996; D.L. Bratton, J. Biol. Chem. 269:22517-22523, 1994; B. Verhoven, et al., Biochim. Biophys. Acta 1104:1523, 1992).

Functional Activity of Recombinant Mouse PL Scramblase. In order to confirm that the cDNA identified

as mouse PL scramblase encodes a protein of similar function to that identified in human, the human and mouse proteins were each expressed in *E. coli*, purified, and reconstituted in proteoliposomes for measurement of PL mobilizing activity. Mouse or human PL scramblase-MBP fusion protein (420 pmoles) was reconstituted into PC/PS liposomes (1  $\mu$ mol total PL), respectively, MBP was removed by digestion of the proteoliposomes with factor Xa, and PL scramblase activity was determined as described under "Experimental Procedures" and plotted as a function of external free  $[Ca^{2+}]$ . The results of this experiment indicate that recombinant mouse PL scramblase mediated a  $Ca^{2+}$ -dependent transbilayer movement of membrane PL with a specific activity and affinity for  $Ca^{2+}$  indistinguishable from that observed for the recombinant human protein.

Mutational Analysis of a Putative Conserved EF-Hand Motif. As noted above, the deduced protein sequence of mouse and human PL scramblase reveals an extensive segment of highly conserved sequence extending through residue Glu<sup>306</sup> (in human; corresponding to Glu<sup>304</sup> in mouse; Fig. 5). The predicted secondary structure through this portion of the protein reveals that it contains two short alpha-helical segments near the C-terminus that are separated by a 12-residue acidic loop. In both proteins (human and mouse), the C-terminal alpha helix represents a predicted transmembrane segment with a strongly-preferred inside-to-outside orientation, whereas sequence

contained within the adjacent 12-residue acidic loop conforms in-part to a consensus sequence that is characteristic of an EF-hand  $\text{Ca}^{2+}$ -binding loop motif (S. Nakayama and R.H. Kretsinger, Annu. Rev. Biophys. Biomol. Struct. 23:473-507, 1994). In this motif, residues in positions 1, 3, 5, 7, 9 and 12 of the loop contribute to octahedral coordination of the  $\text{Ca}^{2+}$  ion, with the residues in position 1[Asp], 3[Asp, Asn, or Ser] and 12 [Asp or Glu] being those most highly conserved.

10 In order to gain insight into whether this segment of the protein might be directly involved in the  $\text{Ca}^{2+}$ -dependent reorganization of membrane PL mediated by PL scramblase, we expressed mutant human PL scramblase with Ala substitutions at positions corresponding to residues  
15 1 (Asp<sup>273</sup>), 3 (Asp<sup>275</sup>), 5 (Phe<sup>277</sup>), 7 (Ile<sup>279</sup>), 9 (Phe<sup>281</sup>), and 12 (Asp<sup>284</sup>) of this putative 12 residue EF-hand loop.

Fig. 6 illustrates PL scramblase activity as a function of mutational analysis of putative EF hand loop motif contained in human PL scramblase. Wild-type (WT)  
20 and mutant constructs of human PL scramblase were expressed as fusion proteins with MBP in *E. coli*, purified, and reconstituted in proteoliposomes. After release of MBP by incubation with factor Xa, PL scramblase activity was assessed (see "Experimental  
25 Procedures"). For each mutant construct, the residues in human PL scramblase that were replaced by Ala are indicated on the abscissa. PL scramblase activity (ordinate) was measured in presence of 2 mM  $\text{CaCl}_2$ , and in

each case was normalized to the activity of WT human PL scramblase ( $11.76 \pm 0.44\%$  of total NBD-PC flipped), with correction for the non-specific transbilayer movement of NBD-PC ( $0.20 \pm 0.08\%$  of total NBD-PC flipped) measured in PL vesicles lacking added protein. Error bars indicate mean  $\pm$  SD of three independent measurements performed with each mutant construct. Fig. 6 illustrates the data of single experiment, representative of two separate experiments so performed.

As illustrated by Fig. 6, Ala substitution at any of these positions reduced PL scramblase function, with mutation at Asp<sup>275</sup> resulting in complete inactivation of the Ca<sup>2+</sup>-dependent response. In those mutant polypeptides showing partial retention of activity, reduced response to Ca<sup>2+</sup> was related in-part to an apparent reduction in avidity for Ca<sup>2+</sup> (Fig. 7).

Fig. 7 illustrates the Ca<sup>2+</sup>-dependence of mutant human PL scramblase. PL scramblase activity of wild-type (WT) and selected mutant constructs of Fig. 6 was determined as described in "Experimental Procedures" and plotted as a function of external free [Ca<sup>2+</sup>]: WT(●); Asp<sup>273</sup>(□); Phe<sup>277</sup>(Δ); Ile<sup>279</sup>(◇); Phe<sup>281</sup>(○); Asp<sup>284</sup>(∇). The data are corrected for non-specific transbilayer migration of NBD-PC in the absence of free [Ca<sup>2+</sup>]. Data of single experiment. The results described in Fig. 7 suggest that residues contained in the putative EF-hand loop spanning Asp<sup>273</sup>-Asp<sup>284</sup> are critical to the function of PL scramblase, presumably for coordination of Ca<sup>2+</sup> as

required to induce the PL transporting state of the protein. It remains to be determined what conformational changes are induced in the polypeptide in the presence of  $\text{Ca}^{2+}$ , including potential reorientation of helical segments flanking the putative  $\text{Ca}^{2+}$  binding loop, that might contribute to the accelerated transbilayer movement of membrane phospholipids.

EXAMPLE 2: Inactivation of Human PL Scramblase by Treatment With the Thiolester Cleaving Reagent, Hydroxylamine (Zhao, et al., Biochemistry 37:6361-6366, 1998).

A. Summary

Incubation of human erythrocyte PL scramblase with hydroxylamine under conditions known to favor hydrolysis of protein cysteinyl-fatty acyl bonds was found to cause near complete loss of PL scramblase's function in promoting movement of PL between membrane leaflets. These data suggest that for normal activity, the PL scramblase polypeptide requires post translational modification through addition of a thiolester-linked fatty acid. Furthermore, these data imply that methods that either prevent cellular acylation of the polypeptide, or that cleave cysteinyl thiolester linkages, will effectively inhibit endogenous PL scramblase activity.

B. Methods

Protein purification. PL scramblase was purified from human erythrocyte ghost membranes as previously

described (F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997).

Treatment with Hydroxylamine. PL scramblase was incubated 1 hour at room temperature in 1 M hydroxylamine, 25 mM octylglucoside, 1 M Tris-HCl at pH 7.4. Match control samples of the protein were identically incubated under these conditions, omitting hydroxylamine. After incubation, samples were dialyzed and reconstituted into PL proteoliposomes for assay of PL  
10 scramblase activity.

Membrane reconstitution and assay. PL scramblase was reconstituted into proteliposomes and activity determined as previously described (F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997):

15 C. Results and Discussion

As shown in Fig. 11, incubation with hydroxylamine resulted in nearly complete inactivation of PL scramblase. Fig. 11 illustrates inactivation of PL scramblase function by treatment with hydroxylamine. The  
20 figure shows loss of functional activity of human erythrocyte PL scramblase due to treatment with the thioester cleaving reagent hydroxylamine. Identical aliquots of human erythrocyte PL scramblase in 50 mM octylglucoside were incubated 1 hour room temperature in  
25 either 1 M hydroxylamine, pH 7.2 (solid bar) or 1 M Tris-HCl, pH 7.2 (open bar). After treatment, each sample was reconstituted into proteoliposomes and PL scramblase activity measured using NBD-phosphatidylcholine as probe

according to methods previously described (Basse, et al.,  
supra, 1996; Stout, et al., supra, 1997; Zhou, et al.,  
supra, 1997). Error bars denote mean  $\pm$  SD of seven  
independent experiments. Significance by paired  
5 students' t-test,  $p < 10^{-6}$ . (See Methods, Example 3.)

Because the conditions of incubation (neutral pH)  
were chosen to favor specific cleavage of cysteinyl  
thioester bonds without disulfide bond reduction, these  
results imply an essential thioester linkage within the  
10 protein. In a membrane-associated protein with  
cytoplasmic Cys residues, such as found in erythrocyte PL  
scramblase, this thioester bond is normally provided by  
palmitic acid in ester linkage to one or more cysteinyl  
thiols. (H. Schroeder, et al., J. Cell Biol. 134:647-660,  
15 1996; M. Stauffenbiel, J. Biol. Chem. 263:13615-13622,  
1988; C.A. Wilcox, et al., Biochemistry 26:1029-1036,  
1987). Whereas the possibility of disulfide reduction by  
hydroxylamine cannot be excluded, it is important to note  
that (1) virtually all cysteine residues in PL scramblase  
20 are normally exposed to cytoplasmic reducing agents such  
as glutathione, and disulfide bond formation is therefore  
not anticipated and (2) The absence of any functionally-  
important disulfide bonds in PL scramblase can be assumed  
based on the retention of normal PL scramblase activity  
25 when the protein was incubated in various reducing  
agents, including dithiothreitol (F. Bassé, et al.,  
supra, 1996; J.G. Stout, et al., supra, 1997). Thus  
these data suggest that PL scramblase polypeptide



requires post-translational modification through addition of a thiolester-linked fatty acid for its normal function in the plasma membrane. Furthermore, these data imply that reagents that either prevent cellular acylation of the polypeptide, or, reagents that cleave cysteinyl thiolester linkages, will effectively inhibit endogenous PL scramblase activity.

The presence of covalently-linked palmitic acid in PL scramblase was directly confirmed by metabolic labeling of the protein with [ $^3\text{H}$ ]-palmitate. Human Raji cells, which we have shown to contain low amounts of endogenous PL scramblase, were transformed with plasmid encoding the human PL scramblase as a fusion protein with green fluorescent protein (GFP), and metabolically labeled with [ $^3\text{H}$ ]-palmitate. Immunoprecipitation with antibody to GFP revealed the covalent incorporation of [ $^3\text{H}$ ] in GFP-PL scramblase fusion protein, whereas no radioactivity was associated with GFP (Fig. 12).

Fig. 12 illustrates that metabolic labeling of PL scramblase with [ $^3\text{H}$ ]-palmitate reveals covalent thioester-linked fatty acid. The presence of cysteinyl-linked palmitate in PL scramblase was confirmed by metabolic labeling of pEGFP-PL scramblase-transfected Raji cells (GFP-PLS) or identically-treated vector-only controls (GFP) with exogenous [ $^3\text{H}$ ]-palmitate. Cells were incubated ( $10^7$  cell/ml, 1 hour,  $37^\circ\text{C}$ ) with  $200\ \mu\text{Ci/ml}$  [ $^3\text{H}$ ]-palmitate (Dupont NEN, spec. act.  $60\ \text{Ci/mmol}$ ) in RPMI-1640 containing 20% dialyzed fetal bovine serum and 5 mM

sodium pyruvate. Cells were then harvested, washed and membrane proteins ( $10^8$  cells/ml) extracted into 1% triton X100, 5 mM EDTA, 20 mM leupeptin, 20 mM M-ethylmaleimide, 10 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride in tris-buffered saline, pH 7.4. Insoluble material was removed by centrifugation and the supernatants precleared by incubation with 10  $\mu$ g/ml normal rabbit IgG and 30  $\mu$ l protein A sepharose (Pharmacia). The GFP and GFP-PLS proteins were then immunoprecipitated overnight with 50  $\mu$ g/ml affinity purified rabbit anti-GFP (Clontech) and staph A sepharose. The beads were exhaustively washed with 1% triton X100 and GFP vector control (Lanes 1 and 2) and the GFP-PL scramblase fusion protein (Lanes 3 and 4) each eluted from staph A sepharose into 5% SDS-PAGE sample buffer. Each sample was divided and incubated 1 hour with either 1 M hydroxylamine, pH 7.2 (+) or 1 M Tris, pH 7.2 (-) and then subjected to polyacrylamide gel electrophoresis under non-reducing conditions (4-20% polyacrylamide; NOVEX). UPPER PANEL: Western blotting was performed with murine monoclonal antibody against human PL scramblase (anti-PLS). MIDDLE PANEL: Western blotting performed with murine monoclonal antibody against green fluorescent protein (anti-GFP; Clontech)). LOWER PANEL shows fluorogram of radioactivity from  $^3\text{H}$ . Fluorography of the SDS-PAGE gel was performed after fixation in 50% ethanol, 5% acetic acid, and the washed gel soaked in Enlightning scintillant (Dupont-NEN) before drying and exposure to preflashed Kodak X-AR5 film (-

80°C, 4 days). Note radioactivity in GFP-PL scramblase (see autoradiography of ~65 kDa protein band, lane 4) that is released from GFP-PL scramblase by treatment with hydroxylamine (see radioactivity at dye front, lane 3).

- 5 Data of single experiment, representative of two independent experiments.

The identity of the covalently-bound [<sup>3</sup>H] in PL scramblase as a thioester-linked [<sup>3</sup>H]-palmitate was confirmed by hydroxylamine cleavage and by thin layer  
10 chromatography (TLC) of the liberated fatty acid (Fig. 13).

Fig.13 illustrates that TLC analysis of [<sup>3</sup>H]-fatty acid in PL scramblase. The GFP-PLS protein band at ~65 kDa of the previous figure was sliced from the wet gel  
15 and the gel soaked in 30% methanol, 10% acetic acid, washed exhaustively with 50% methanol, and then lyophilized. To liberate covalently-bound fatty acids, the dried gel was incubated overnight in 1.5N NaOH. Following acidification with HCl (pH<2), the released  
20 fatty acids (1 ml) were extracted by addition of 3.75 ml chloroform:methanol (1:2 v:v). To separate the fatty acids into a chloroform phase, 1.25 ml chloroform and 1.25 ml H<sub>2</sub>O were then added, and the bottom layer recovered and dried under nitrogen. The dried pellet was  
25 taken up in chloroform:methanol (2:1, v:v) and spotted onto KC18 silica gel (Whatman 4801-425). The chromatograph was developed in acetonitrile:acetic acid (9:1, v:v) and air dried. The dried plate was sprayed

with Enhance (Dupont-NEN) and radioactivity was detected by fluorography on preflashed Kodak XAR-5 film (-80°C, 4 days). Radioactivity liberated from GFP-PLS (lane 2) is compared to matched gel slice from GFP vector control (lane 3), using [<sup>3</sup>H]-palmitate as standard (standards applied in lanes 1 and 4). All samples were spotted at position OR; SF indicates position of the solvent front. Data of single experiment.

Thus, PL scramblase is palmitoylated at one or more cysteine residues. We also have evidence that such acylation is required for normal function of PL scramblase protein. As noted above, removal of palmitate through hydrolysis of thioester bonds with hydroxylamine was found to abolish PL scramblase's phospholipid mobilizing function (Fig. 11).

EXAMPLE 3: Plasma Membrane Expression of Phospholipid Scramblase Regulates Ca<sup>2+</sup> Induced Movement of Phosphatidylserine to the Cell Surface: Alteration of Phosphatidylserine Exposure In Human Lymphoblasts Through Stable Transfection with PL Scramblase cDNA.

A. Summary

In order to determine whether PL scramblase is responsible for the rapid movement of PS from inner-to-outer plasma membrane leaflets in other cells exposed to elevated cytosolic [Ca<sup>2+</sup>]<sub>c</sub>, we analyzed how induced movement of PS to the surface related to cellular content of PL scramblase. Exposure to Ca<sup>2+</sup> ionophore A23187 resulted in rapid PS exposure in those cells high in PL

scramblase (K-562, HEL, 293T, and EBV-transformed lymphocytes), whereas this response was markedly attenuated in cells with low amounts of the protein (Raji, MOLT-4, HL-60). To confirm this apparent correlation between PL scramblase expression and PS egress at elevated  $[Ca^{2+}]_c$ , Raji cells were transfected with PL scramblase cDNA in pEGFP-C2, and stable transformants expressing various amounts of rGFP-PL scramblase fusion protein obtained. Clones expressing rGFP-PL scramblase showed plasma membrane-localized fluorescence and elevated PL scramblase antigen whereas clones expressing rGFP alone (transfected with pEGFP-C2 without insert) showed only cytoplasmic fluorescence and served as controls. In absence of ionophore, expression of rGFP-PL scramblase had no effect on cell viability or background PS exposure. In response to A23187, clones expressing GFP-PL scramblase exhibited markedly accelerated movement of PS to the cell surface when compared to A23187-treated clones expressing GFP with PS movement to the cell surface increasing with amount of rGFP-PL scramblase expressed. These data indicate that transfection with PL scramblase cDNA promotes  $[Ca^{2+}]_c$ -dependent movement of PS to the cell surface and suggest that this protein normally mediates redistribution of plasma membrane phospholipids in activated, injured, or apoptotic cells exposed to elevated  $[Ca^{2+}]_c$ .

B. Materials and Methods

Materials. All restriction enzymes were from New England BioLabs, Inc. (Beverly, MA). Klentaq polymerase and pEGFP-C2 vector were from CLONTECH Laboratories (Palo Alto, CA). Bovine coagulation factor Va (FVa), factor Xa (FXa), prothrombin and dansylarginine-N-(3-ethyl-1,5-pentanedyl)amide were from Haematologic Technologies, Inc. (Essex Junction, VT). Chromogenic thrombin substrate S2238 was from DiaPharma Group, Inc. (Franklin, OH). Human  $\alpha$ -thrombin was a generous gift from Dr. John W. Fenton (Albany, NY). OPTI-MEM and geneticin were from Life Technologies (Gaithersburg, MD). Fetal bovine serum, RPMI 1640, Cell Dissociation Solution, Hank's Balanced Salt Solution (HBSS), Protein A Sepharose-CL4B, leupeptin, and BSA were from Sigma Chemical Co. (St. Louis, MO). UltraLink Iodoacetyl resin and SuperSignal ULTRA Chemiluminescence Kit were from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

Cell culture: Human cancer cell lines erythroleukemic HEL, promyelocytic leukemia HL-60, chronic myelogenous leukemia K562, lymphoblastic leukemia MOLT-4, acute T-cell leukemia Jurkat, Burkitt's lymphoma Raji, and megakaryocytic DAMI were from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 containing 10% fetal bovine serum. EBV-transformed cell line W9 established from peripheral B-lymphocytes of

a normal donor was maintained as previously described (H. Kojima, et al., 1994).

Antibodies: Anti-GFP: murine monoclonal antibody against green fluorescent protein (GFP) was from CLONTECH Laboratories. Anti-FVa: murine monoclonal antibody V237 reactive against human or bovine factor Va light chain was the generous gift of Dr. Charles T. Esmon (Oklahoma Medical Research Fndn, Oklahoma City, OK). Anti-PL Scramblase-E306-W318: Rabbit antibody raised against the carboxyl terminal peptide sequence E306-W318 of human PL  
10       scramblase has previously been described (Q. Zhou, et al., supra, 1997). The IgG fraction was isolated on protein A-Sepharose-CL4B and the peptide-reactive antibody purified by affinity chromatography on peptide  
15       [Cys]-ESTGSQEQKSGVW (SEQ ID NO:5) coupled to UltraLink Iodoacetyl resin.

Plasmid Construction: Human PL scramblase cDNA insert was released from plasmid pMAL-C2-PL scramblase (Q. Zhou, et al., supra, 1997) by double cutting with  
20       EcoRI and SalI, respectively, and then ligated into pEGFP-C2 vector using the same restriction site. The pEGFP-C2-PL scramblase plasmid was amplified from single clones in *E. coli* strain Top 10, and the orientation and reading frame of the insert confirmed by sequencing on an  
25       ABI DNA Sequencer Model 373 Stretch (Perkin Elmer-Applied Biosystems, Foster City, CA) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit.

Transfection of Raji cells with pEGFP-PL scramblase.

1.6 x 10<sup>7</sup> Raji cells were electroporated with 160 µg plasmid DNA (pEGFP-C2-PL scramblase or pEGFP-C2) in a total volume of 0.8 ml OPTI-MEM, using Gene Pulse  
5 Electroporator (Bio-Rad Laboratories, Hercules, CA) set at 450 V, 500°F. After 48 hours in culture, 1.5 mg/ml geneticin was added to the medium and continuously maintained for 4 weeks. Stable transformants exhibiting GFP fluorescence were sorted by flow cytometry (FACStar,  
10 Becton-Dickinson Immunocytometry Systems, San José, CA) using an FL1 sorting gate. The FL1-positive cells were dilutionally cloned in 96 well culture plates. pEGFP-PL scramblase transformants expressing the 62 kDa GFP-PL scramblase fusion protein were identified by Western  
15 blotting with anti-GFP and with anti-PL-306-W318 antibodies. Western blotting of pEGFP-C2 transformants (without insert) confirmed presence of 27 kDa GFP. Clones expressing various amounts of GFP-PL scramblase were each expanded for functional assay, along with  
20 comparable GFP-expressing clones serving as controls.

Fluorescence Microscopy. Cell clones transfected with pEGFP-C2-PL scramblase or pEGFP-C2 were deposited on glass microscopy slides using a Cytospin 3 (Shandon, Inc., Pittsburgh, PA). Phase contrast and fluorescence  
25 microscopy was performed with a ZEISS AXIOSKOP microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence, and images were recorded with a MC100 camera system. The exposure times for photography of



fluorescence was 80-200 seconds under automatic control using Kodak Ektachrome 1600 film.

Western Blot Analysis. Western blotting of GFP and PL scramblase antigens was performed using  $1.5 \times 10^6$  cells per lane. After washing in HBSS, supernatants were removed, and the cell pellets extracted with 2% (v/v) NP-40 in 5 mM EDTA, 50 mM benzamidine, 50 mM N-ethyl maleimide, 1 mM phenylmethanesulfonyl fluoride, 1 mM leupeptin in HBSS. After removal of insoluble material (250,000 x g, 30 minutes, 4°C), the samples were denatured at 100°C in 10% (w/v) SDS sample buffer containing 2%  $\beta$ -mercaptoethanol. Following SDS-PAGE and transfer to nitrocellulose, the blocked membrane was incubated with either 10  $\mu$ g/ml of rabbit anti-PL scramblase-E306-W318, or 1/10,000 dilution of mouse anti-GFP. The blots were developed with the horseradish peroxidase conjugate of either goat anti-rabbit IgG or goat anti-mouse IgG, respectively, using SuperSignal ULTRA chemiluminescence.

Measurement of cell surface PS. Calcium ionophore-induced exposure of PS on the surface of all cell lines analyzed was detected by the specific binding of coagulation factor Va (light chain) as previously described (P.J. Sims, *et al.*, J. Biol. Chem. 263:18205-18212, 1988; H. Kojima, *et al.*, J. Clin. Invest. 94:2237-2243, 1994). Briefly, cells were washed twice to remove serum proteins and suspended ( $2 \times 10^6$  cells/ml) at 37°C in RPMI 1640 supplemented with 0.1% BSA, 20 mM HEPES, and

adjusted to 1.2 mM free  $[Ca^{2+}]$ . At time=0, A23187 (0 or 2  $\mu$ M final concentration) was added from 1 mM stock solution in DMSO, and at times indicated in figure legends, the reaction was stopped by addition of 6 mM EGTA. PS exposed on the cell surface at each time point was detected by incubating (10 minutes, room temperature) 50  $\mu$ l of the cell suspension with 10  $\mu$ g/ml FVa, followed by 10  $\mu$ g/ml anti-FVa, to detect the cell-bound FVa light chain. After staining with 10  $\mu$ g/ml Tri-Color conjugated goat anti-mouse IgG (CALTAG Laboratories, Burlingame, CA), single-cell fluorescence was quantitated by flow cytometry (FL3 channel, FACScan, Becton Dickinson Immunocytometry Systems). Use of Tri-Color conjugate to detect cell-bound FVa enabled simultaneous measurement of cell-associated GFP fluorescence in cell lines transformed with the pEGFP-C2 expression plasmid (fluorescence of GFP detected in FL1 channel). In experiments in which cell lysis was monitored by uptake of propidium iodide, cells were stained for bound FVa with FITC-conjugate of goat anti-mouse IgG (FL1 channel) substituting for Tri-Color conjugate, and propidium iodide was detected in FL3 channel. Propidium iodide (0.5  $\mu$ g/ml) was added immediately before dilution for flow cytometry.

Prothrombinase Assay. Prothrombinase activity of Raji cells was determined by modification of methods previously described for platelets, using the chromogenic thrombin substrate S2238 (P.J. Sims, *et al.*, *supra*,

1988). Briefly,  $1 \times 10^5$  Raji cells (transfected with either pEGFP-C2 or pEGFP-C2-PL scramblase) were suspended in 200  $\mu$ l HBSS containing 1% BSA in the presence of 2 nM FVa, 1.4  $\mu$ M prothrombin 2.5 mM  $\text{CaCl}_2$ , and 4  $\mu$ M dansylarginine-N-(3-ethyl-1,5-pentanediy1)amide (to inhibit feed-back activation by thrombin), and incubated at 37°C.  $\text{Ca}^{2+}$ -ionophore A23187 (2  $\mu$ M), or DMSO (as solvent control) was added, and prothrombin conversion was initiated by addition of 2 nM Fxa. Thrombin generation was stopped after 2 minutes by dilution into 10 mM EGTA and samples were stored on ice. Aliquots were transferred to a 96-well plate, and thrombin generated was assayed in TBS containing 1% BSA in presence of 150  $\mu$ M S2238 by monitoring time-dependent changes in absorbance at 405 nm using a Thermo<sub>max</sub> plate reader (Molecular Devices, Sunnyvale, CA). Thrombin activity was calculated using purified thrombin as standard.

### C. Results

Analysis of PL scramblase in various human cell lines. Proteoliposomes reconstituted with erythrocyte PL scramblase exhibit accelerated transbilayer movement of fluorescent phospholipids in response to added  $\text{Ca}^{2+}$ , similar to the observed effect of calcium on the endofacial surface of the red cell membrane (Q. Zhou, et al., supra, 1997; J.G. Stout, et al., J. Clin. Invest. 99:2232-2238, 1997; Bassé, et al., J. Biol. Chem. 271:17205-17210, 1996). In order to determine whether this same protein is responsible for mediating the

accelerated egress of plasma membrane PS that is observed under conditions of elevated cytosolic  $[Ca^{2+}]_c$ , we undertook to determine whether the level of expression of PL scramblase in various human cell lines correlated to the induced movement of PS to the surface of these cells. When challenged with a calcium ionophore, human cell lines exhibit considerable differences in the extent to which PS is mobilized to the cell surface. Among the cells tested, Raji, HL60, and Dami were notably unresponsive to A23187, whereas HEL, W9 (an EBV-transformed normal B-lymphocyte), and Jurkat showed notably robust responses. This apparent cell type-specific variability in response to induced elevation of  $[Ca^{2+}]_c$  was consistently maintained through many months of passage in culture, suggesting it reflected an inherent property of each cell line. As shown in Fig. 8, we also observed considerable differences in the content of PL scramblase protein among these various cell lines, and the sensitivity of these various cell lines to induced exposure of plasma membrane PS (*lower panel*) generally correlated with the amount of cellular PL scramblase protein detected by Western blotting (*upper panel*): Those cell lines that were most responsive to induced elevation of  $[Ca^{2+}]_c$  (HEL, W9, Jurkat) also expressed greatest amounts of PL scramblase antigen, whereas cell lines with attenuated response to  $[Ca^{2+}]_c$  (Raji, HL60, Dami) contained relatively little of this protein. Cell lines Molt-4 and K562 showed intermediate responses to

elevated  $[Ca^{2+}]_c$  and expressed intermediate levels of PL scramblase antigen.

Fig. 8 depicts western blot analysis of PL scramblase in various human cell lines. Constitutive expression of PL scramblase was analyzed in the human cell lines indicated. Upper Panel: Results obtained by Western blotting with antibody specific for PL scramblase carboxyl terminal residues E306-W318 (see *Materials & Methods*). Each lane contains the total protein extract of  $1.5 \times 10^6$  cells. Lower Panel: Cumulative results of three separate experiments performed as follows: The cells indicated were washed and suspended at  $37^\circ C$  in the presence of 1.2 mM free  $Ca^{2+}$ , and 2  $\mu M$  A23187 was added. At times shown (*abscissa*), EGTA was added and cells analyzed for surface exposed PS as detected by cell-bound FVa light chain (see *Materials and Methods*). Data plotted represent the mean increase ( $\pm$  SD) in number of cells that stained positive for surface PS after 5 minutes incubation with ionophore, after correction for initial background of PS-positive cells before addition of ionophore (time=0). Background number of cells that exposed PS in absence of ionophore was always <15% except in case of HEL, where this background ranged between 15-30%.

These relatively large differences in cell line-specific expression of this protein was also consistently observed despite repeated passage in culture, and was found to correspond to marked differences in level of

specific mRNA as detected by Northern blotting with PL scramblase cDNA (Q. Zhou, *et al.*, *supra*, 1997), and data not shown). We also noted that those cell lines with the highest content of PL scramblase generally exhibited a higher background of PS exposed on the surface in absence of added ionophore. This was most notable for HEL for which approximately 15-30% of the cells were consistently found to expose PS prior to addition of A23187 (see Discussion).

10        Membrane changes underlying ionophore response. In order to determine whether the increase in PS exposure in ionophore-treated cells reflected facilitated movement of PS from inner to outer leaflets of the plasma membrane, or, greater sensitivity of the plasma membrane to lytic  
15        disruption, FVa binding to the cell surface was monitored simultaneously with uptake of propidium iodide, as a measure of cell lysis. As illustrated for the human B-lymphocyte lines W9 (high content of PL scramblase) and Raji (low content of PL scramblase), the induced movement  
20        of PS to the cell surface was found to precede uptake of propidium iodide, suggesting that the elevation of  $[Ca^{2+}]_i$  induces a collapse of transmembrane PL asymmetry before onset of lysis. In the case of Raji cells which are virtually devoid of PL scramblase (see Fig. 8), a general  
25        insensitivity of the plasma membrane to either ionophore-induced PS exposure or to lysis was also apparent.

Transfection of the Raji cell line with pEGFP-C2-PL scramblase. In order to confirm that the extent to which

PS moves to the cell surface with elevation of  $[Ca^{2+}]_i$ . actually depends upon the plasma membrane content of PL scramblase, we stably transformed Raji, a cell line exhibiting low endogenous PL scramblase expression by transfection with plasmid pEGFP-C2-PL scramblase. This plasmid expresses PL scramblase as a fusion protein with green fluorescent protein (GFP), facilitating flow cytometric sorting of transformants for subsequent cloning and detection of the expressed recombinant protein in selected clones. The decision to attach GFP to the amino terminus of PL scramblase was based on prior evidence that the carboxyl terminus of the protein is membrane inserted and essential for function, and the observation that other amino terminal fusion constructs of PL scramblase expressed in *E. coli* retained the same activity of the unmodified PL scramblase polypeptide when reconstituted in proteoliposomes (Q. Zhou, et al., supra, 1997), and unpublished data). The expression of the full-length GFP-PL scramblase fusion protein in selected transformed clones was confirmed by Western blotting with antibody specific for GFP, and with antibody raised against peptide sequence of the carboxyl terminus of human PL scramblase. As illustrated by fluorescence micrographs shown in Fig. 9, clones that expressed the GFP-PL scramblase fusion protein showed a distinct rim appearing pattern of fluorescence, consistent with trafficking of GFP-PL scramblase to the plasma membrane. Fig. 9 illustrates fluorescence micrographs of GFP-PL

scramblase transformed Raji cells. Fluorescence photomicrography of GFP fluorescence expressed in the transformed Raji clones was performed as described in *Materials and Methods*. Fig 9A shows fluorescence of cells expressing GFP; Fig. 9B shows cells transfected with pEGFP-C2-PL scramblase plasmid and expressing GFP-PL scramblase fusion protein. Data of single experiment, representative of results obtained for all clones transfected with either pEGFP-C2 or pEGFP-C2-PL

10 scramblase. By contrast, clones that expressed GFP alone exhibited diffuse fluorescence throughout the cytoplasm, with no obvious staining of the plasma membrane. These data provide the first direct evidence that PL scramblase cDNA encodes a protein that predominantly trafficks to

15 the plasma membrane under normal conditions of cell growth.

Analysis of PS mobilizing function in GFP-PL scramblase transformants. After geneticin selection, clonal populations of transformed Raji cells expressing

20 comparable levels of either GFP-PL scramblase or GFP (transformed with pEGFP-C2 lacking insert) were analyzed for their capacity to mobilize PS to the cell surface. In response to an A23187-induced elevation of  $[Ca^{2+}]_i$ , transformants expressing the GFP-PL scramblase fusion

25 construct showed a marked increase in both the rate and extent that PS became exposed on the cell surface, when compared to either the identically-treated parental Raji cell line or to GFP-expressing clones transformed with



pEGFP-C2 vector alone. As was also evident from these data, in the absence of ionophore, we consistently noted a small but reproducible increase in the background level of PS exposure in transformants expressing GFP-PL  
5 scramblase protein, when compared to either the parental Raji cell lines or to GFP-expressing clones transformed with vector alone.

Induction of membrane procoagulant function through expression of PL scramblase. In order to confirm that  
10 the increased expression of FVa binding sites detected upon activation of GFP-PL scramblase transformed clones reflected an increase in the procoagulant (clot-promoting) properties of the plasma membrane of these cells, the capacity of GFP-PL scramblase transformed  
15 cells to provide catalytic membrane surface for the prothrombinase (FVaXa) enzyme complex was compared to clones expressing GFP alone. These data confirmed that expression of recombinant PL scramblase in the Raji cell line was also accompanied by an increase in cell capacity  
20 to catalyze the prothrombinase reaction upon entry of calcium into the cytosol.

Level of expression of PL scramblase regulates capacity of to mobilize PS to the cell surface. In order to confirm the apparent correlation between endogenous  
25 cell content of PL scramblase and plasma membrane sensitivity to elevated  $[Ca^{2+}]_c$  that is evident when different human cell lines are compared (see Fig. 8), we analyzed multiple Raji clones that were stably

transfected with either GFP-PL scramblase or with GFP vector alone (Fig. 10). Fig. 10 illustrates that the level of expression of PL scramblase determines plasma membrane sensitivity to intracellular  $\text{Ca}^{2+}$ . The relationship between level of recombinant protein expressed (GFP fluorescence detected in FL1 channel; *abscissa*) and numbers of cells that expose PS after 2 minutes incubation with A23187 (*ordinate*) is plotted for multiple transformed Raji clones. Analysis was gated to include only those cells distinctly positive for GFP fluorescence (FL1 channel), and cell-bound FVa was stained with Tri-color conjugate and detected in FL3 channel (see *Materials and Methods*). Analysis was performed on all cells positive for GFP fluorescence.

Open symbols indicate individual clones stably transformed by transfection with pEGFP-C2; closed symbols indicate individual clones stably transformed with pEGFP-PL scramblase. Data of single experiment, representative of three so performed. These experiments confirm that the capacity of GFP-PL scramblase transformants to mobilize PS to the cell surface generally correlates with the amount of the expressed GFP-PL scramblase fusion protein, whereas this cell response to increased  $[\text{Ca}^{2+}]_i$  is unaffected by cell content of GFP. In addition to confirming the role of PL scramblase in the plasma membrane response to  $[\text{Ca}^{2+}]_i$ , these data suggest that the capacity to mobilize PS to the cell surface and thereby support plasma clotting in activated, injured or

apoptotic cells exposed to elevated  $[Ca^{2+}]_c$  can be altered by changing the level of expression of PL scramblase expressed in the plasma membrane.

### Discussion

5           These results provide the first evidence that the PL scramblase protein identified in the erythrocyte membrane and implicated in  $[Ca^{2+}]_c$ -induced remodeling of membrane phospholipids actually functions to induce accelerated transbilayer movement of plasma membrane phospholipid in  
10 human cells that express this protein. Our results also confirm that the level of expression of plasma membrane PL scramblase can determine the extent to which PS is mobilized to the cell surface upon elevation of  $[Ca^{2+}]_c$ , and suggest that this protein normally functions to  
15 mediate the redistribution of plasma membrane phospholipids in response to the entry of calcium into the cytosol. Furthermore, these data provide the first indication that the movement of PS and other procoagulant aminophospholipids from plasma membrane inner leaflet to  
20 the cell surface can be manipulated by selectively altering the level of expression of a particular cellular protein, either through direct transfection with the PL scramblase cDNA, or potentially, by another intervention affecting cellular expression of functional PL scramblase  
25 protein.

Whereas these experiments suggest that direct activation of plasma membrane PL scramblase is responsible for the increased cell surface exposure of PS

that is observed in various activated, injured or apoptotic cells exposed to elevated  $[Ca^{2+}]_c$ , we cannot exclude the possibility that there are other cellular components that contribute to the accelerated movement of PS from inner to outer plasma membrane leaflet under these conditions. In particular, whereas PL scramblase has been shown to mediate the bidirectional movement of PS and other phospholipids between membrane leaflets, it has been suggested that there is also a PS-selective pathway in the platelet plasma membrane, designated "PS floppase", which mediates vectorial movement of PS from inner to outer plasma membrane leaflet (P. Gaffet, et al., Biochemistry 34:6762-6769, 1995). Experimental evidence for the existence of this vectorial and headgroup-selective PS floppase pathway in platelet or other cell membranes remains controversial (R.F.A. Zwaal, et al., supra, 1997; P. Williamson, et al., Biochemistry 31:6355-6360, 1995; C.-P. Chang, et al., J. Biol. Chem. 268:7171-7178, 1993), and awaits identity of a  $[Ca^{2+}]_c$ -activated and PS-selective transporter that is distinct from the plasma membrane PL scramblase found in platelets and erythrocytes, a protein that does not exhibit apparent selectivity for the PS headgroup (J.G. Stout, et al., supra, 1997; P. Comfurius, et al., Biochemistry 35,7631-7634, 1996; F. Bassé, et al., supra, 1996).

In addition to conferring increased sensitivity of the plasma membrane to ionophore-induced elevation of  $[Ca^{2+}]_c$ , we generally observed a higher background of PS

exposure (in absence of ionophore) in those transfected cell clones expressing large amounts of the GFP-PL scramblase fusion protein. This elevated background PS exposure was also observed in the case of untreated HEL, the cell line containing the highest endogenous content of PL scramblase. Although we suspect that this increased background reflects the enhanced sensitivity of the plasma membrane of these cells to any adventitial elevation of  $[Ca^{2+}]_e$  during cell processing for assay, we cannot exclude the possibility that these cells are also inherently more fragile due to the large amounts of PL scramblase that is inserted into the plasma membrane.

While the movement of plasma membrane PS to the cell surface at elevated  $[Ca^{2+}]_e$  can be demonstrated in a variety of cells and tissues (R.F.A. Zwaal, et al., supra, 1997; P. Devaux, supra, 1991), we detect marked differences in the levels of PL scramblase mRNA and protein among different human cell types, which is generally reflected by corresponding differences in sensitivity to this  $[Ca^{2+}]_e$ -induced collapse of plasma membrane PL asymmetry (see Fig. 8, and Q. Zhou, et al., supra, 1997). Although the transcriptional regulation of the PL scramblase gene remains to be determined, it is of interest to note that such cell or tissue-specific differences in PL scramblase expression has the potential to significantly affect the biological properties of the cell. In particular, we note that the content of PL scramblase in human platelet is approximately 10-fold

greater than that of the erythrocyte, which is consistent with the respective PS-mobilizing potential and different roles of these two cells in contributing procoagulant membrane surface for thrombin generation during blood clotting (Q. Zhou, et al., supra, 1997). In addition to the relatively high levels of PL scramblase identified in circulating human platelets, this protein was also most abundant in the cell line HEL, whereas only small amounts of this protein (and low PL scramblase activity) was detected for Dami (Fig. 8), two human cancer cell lines exhibiting partial megakaryocytic-like properties. It is also noteworthy that several of the lymphoma-derived cell lines (e.g., Raji, MOLT-4) express considerably reduced levels of PL scramblase, and also show distinctly attenuated PS exposure in response to elevated  $[Ca^{2+}]_c$ , when compared to either peripheral blood leukocytes or to EBV-transforms of normal lymphocytes (Fig. 8). The collapse of plasma membrane phospholipid asymmetry is a relatively early event in apoptosis of lymphocytes and other cells, and the consequent exposure of PS on the cell surface is thought to contribute to phagocytic removal of such cells by scavenger macrophages (V.A. Fadok, et al., J. Immunol. 148:2207-2216, 1992; B. Verhoven, et al., J. Exp. Med. 182:1597-1601, 1995). It is therefore of interest to consider whether the apparent resistance of certain lymphoma-derived cell lines to such  $[Ca^{2+}]_c$ -induced remodeling of plasma membrane phospholipids might contribute to the proliferative

potential and *in vivo* survival of these or other transformed cells.

EXAMPLE 4: Induction of Programmed Cell Death is Related to the Cellular Content of PL Scramblase

5 A. Summary

Human cell lines expressing various levels of PL  
scramblase protein were exposed to agents or conditions  
of cell culture known to promote apoptosis (programmed  
cell death). Analysis of these cell lines revealed that  
10 the number of cells induced to undergo apoptosis  
generally correlated with the level of expression of PL  
scramblase protein, with those cell lines expressing high  
levels of PL scramblase being most sensitive to induction  
of apoptosis while cell lines expressing low levels of PL  
15 scramblase being most resistant to induction of  
apoptosis. The human Burkitts B-lymphoma cell line Raji  
was found to express low levels of PL scramblase and to  
be markedly resistant to induction of apoptosis.  
Transfection of Raji cells with plasmid containing cDNA  
20 for PL scramblase resulted in increased sensitivity of  
this cell to the induction of apoptosis, demonstrating  
that expression of PL scramblase in a normally apoptosis-  
resistant cancer cell line promotes programmed death of  
the cancer cell.

25 B. Materials and Methods

Materials. All restriction enzymes were from New  
England BioLabs, Inc. (Beverly, MA). Klentaq polymerase  
and pEGFP-C2 vector were from CLONTECH Laboratories (Palo  
Alto, CA). Bovine coagulation factor Va (Fva). OPTI-MEM

and geneticin were from Life Technologies (Gaithersburg, MD). Fetal bovine serum, RPMI 1640, Cell Dissociation Solution, Hank's Balanced Salt Solution (HBSS), Protein A Sepharose-CL4B, leupeptin, and BSA were from Sigma Chemical Co. (St. Louis, MO). UltraLink Iodoacetyl resin and SuperSignal ULTRA Chemiluminescence Kit were from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

Antibodies: Anti-GFP: murine monoclonal antibody against green fluorescent protein (GFP) was from CLONTECH Laboratories. Anti-FVa: murine monoclonal antibody V237 reactive against human or bovine factor Va light chain was the generous gift of Dr. Charles T. Esmon (Oklahoma Medical Research Fndn, Oklahoma City, OK). Anti-PL Scramblase-E306-W318: Rabbit antibody raised against the carboxyl terminal peptide sequence E306-W318 of human PL scramblase has previously been described (Q. Zhou, et al., supra, 1997). The IgG fraction was isolated on protein A-Sepharose-CL4B and the peptide-reactive antibody purified by affinity chromatography on peptide [Cys]-ESTGSQEQKSGVW (SEQ ID NO:5) coupled to UltraLink Iodoacetyl resin. The IgG fraction of anti-human FAS ligand was from Clontech (Sunnyvale, CA).

Cell culture: Human cancer cell lines erythroleukemic HEL, promyelocytic leukemia HL-60, chronic myelogenous leukemia K562, lymphoblastic leukemia MOLT-4, acute T-cell leukemia Jurkat, Burkitt's lymphoma Raji, and megakaryocytic DAMI were from American Type



Culture Collection (Rockville, MD) and cultured in RPMI 1640 containing 10% fetal bovine serum. EBV-transformed cell line W9 established from peripheral B-lymphocytes of a normal donor was maintained as previously described (H. Kojima, et al., 1994).

Plasmid Construction: Human PL scramblase cDNA insert was released from plasmid pMAL-C2-PL scramblase (Q. Zhou, et al., supra, 1997) by double cutting with *EcoRI* and *SallI*, respectively, and then ligated into pEGFP-C2 vector using the same restriction site. The pEGFP-C2-PL scramblase plasmid was amplified from single clones in *E. coli* strain Top 10, and the orientation and reading frame of the insert confirmed by sequencing on an ABI DNA Sequencer Model 373 Stretch (Perkin Elmer-Applied Biosystems, Foster City, CA) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit.

Transfection of Raji cells with pEGFP-PL scramblase.  $1.6 \times 10^7$  Raji cells were electroporated with 160  $\mu$ g plasmid DNA (pEGFP-C2-PL scramblase or pEGFP-C2) in a total volume of 0.8 ml OPTI-MEM, using Gene Pulse Electroporator (Bio-Rad Laboratories, Hercules, CA) set at 450 V, 500 $^{\circ}$ F. After 48 hours in culture, 1.5 mg/ml geneticin was added to the medium and continuously maintained for 4 weeks. Stable transformants exhibiting GFP fluorescence were sorted by flow cytometry (FACStar, Becton-Dickinson Immunocytometry Systems, San José, CA) using an FL1 sorting gate. The FL1-positive cells were dilutionally cloned in 96 well culture plates. pEGFP-PL

scramblase transformants expressing the 62 kDa GFP-PL  
scramblase fusion protein were identified by Western  
blotting with anti-GFP and with anti-PL-306-W318  
antibodies. Western blotting of pEGFP-C2 transformants  
5 (without insert) confirmed presence of 27 kDa GFP.  
Clones expressing various amounts of GFP-PL scramblase  
were each expanded for functional assay, along with  
comparable GFP-expressing clones serving as controls.

Fluorescence Microscopy. Cell clones transfected  
10 with pEGFP-C2-PL scramblase or pEGFP-C2 were deposited on  
glass microscopy slides using a Cytospin 3 (Shandon,  
Inc., Pittsburgh, PA). Phase contrast and fluorescence  
microscopy was performed with a ZEISS AXIOSKOP microscope  
(Carl Zeiss, Inc., Thornwood, NY) equipped for  
15 epifluorescence, and images were recorded with a MC100  
camera system. The exposure times for photography of  
fluorescence was 80-200 seconds under automatic control  
using Kodak Ektachrome 1600 film.

Western Blot Analysis. Western blotting of GFP and  
20 PL scramblase antigens was performed using  $1.5 \times 10^6$  cells  
per lane. After washing in HBSS, supernatants were  
removed, and the cell pellets extracted with 2% (v/v) NP-  
40 in 5 mM EDTA, 50 mM benzamidine, 50 mM N-ethyl  
maleimide, 1 mM phenylmethylsulfonyl fluoride, 1 mM  
25 leupeptin in HBSS. After removal of insoluble material  
(250,000 x g, 30 minutes, 4°C), the samples were  
denatured at 100°C in 10% (w/v) SDS sample buffer  
containing 2%  $\beta$ -mercaptoethanol. Following SDS-PAGE and

transfer to nitrocellulose, the blocked membrane was incubated with either 10  $\mu$ g/ml of rabbit anti-PL scramblase-E306-W318, or 1/10,000 dilution of mouse anti-GFP. The blots were developed with the horseradish  
5 peroxidase conjugate of either goat anti-rabbit IgG or goat anti-mouse IgG, respectively, using SuperSignal ULTRA chemiluminescence.

Induction of apoptosis: Apoptosis was induced either by (1) addition of 60  $\mu$ M etoposide to the cell  
10 culture (2) by removal of fetal bovine serum from the culture medium (serum starvation). Cells were maintained in sterile culture flasks in 37°C incubator under each of these conditions for 72 hours, during which cell aliquots were obtained for assay (times indicated in figure  
15 legends).

Assays for Apoptosis: The induction of apoptosis was detected by (1) egress of PS to the cell surface (2) activation of cellular caspase enzymes (3) cleavage of nuclear DNA. Egress of PS to the cell surface was  
20 measured by the binding of Factor Va (light chain) or by the binding of FITC-annexin V, detected by flow cytometry. Cleavage of nuclear DNA was measured either by a decrease in propidium iodide staining of the nucleus, according to published methods (ref), or by the  
25 measured incorporation of FITC-dUTP into 5'-ends of chromosomal DNA, catalyzed by the enzyme terminal deoxyribose transferase (TUNEL Assay Kit, Clontech) according to the manufacturer's protocol. In each assay,

fluorescence was detected by flow cytometry (FACScan, Becton Dickinson), using the FL1 channel to detect FITC or the FL3 channel to detect propidium iodide staining of the nucleus. Forward and side scatter gates were set to  
5 exclude cell debris.

Measurement of cell surface PS. Exposure of PS on the surface of all cell lines analyzed was detected by the specific binding of coagulation factor Va (light chain) as previously described (P.J. Sims, et al., J. Biol. Chem. 263:18205-18212, 1988; H. Kojima, et al., J. Clin. Invest. 94:2237-2243, 1994). Briefly, cells were  
10 washed twice to remove serum proteins and suspended ( $2 \times 10^6$  cells/ml) at 37°C in RPMI 1640 supplemented with 0.1% BSA, 20 mM HEPES, and adjusted to 1.2 mM free  $[Ca^{2+}]$ . At  
15 time=0, A23187 (0 or 2  $\mu$ M final concentration) was added from 1 mM stock solution in DMSO, and at times indicated in figure legends, the reaction was stopped by addition of 6 mM EGTA. PS exposed on the cell surface at each  
20 time point was detected by incubating (10 minutes, room temperature) 50  $\mu$ l of the cell suspension with 10  $\mu$ g/ml FVa, followed by 10  $\mu$ g/ml anti-FVa, to detect the cell-bound FVa light chain. After staining with 10  $\mu$ g/ml Tri-Color conjugated goat anti-mouse IgG (CALTAG  
Laboratories, Burlingame, CA), single-cell fluorescence  
25 was quantitated by flow cytometry (FL3 channel, FACScan, Becton Dickinson Immunocytometry Systems). Use of Tri-Color conjugate to detect cell-bound FVa enabled simultaneous measurement of cell-associated GFP

fluorescence in cell lines transformed with the pEGFP-C2 expression plasmid (fluorescence of GFP detected in FL1 channel). In experiments in which cell lysis was monitored by uptake of propidium iodide, cells were stained for bound FVa with FITC-conjugate of goat anti-mouse IgG (FL1 channel) substituting for Tri-Color conjugate, and propidium iodide was detected in FL3 channel. Propidium iodide (0.5  $\mu$ g/ml) was added immediately before dilution for flow cytometry.

10 C. Results

Role of PL scramblase in PS mobilization under conditions of induced apoptosis. As it appeared that the cellular content of PL scramblase determines the sensitivity of the plasma membrane to accelerated transbilayer migration of PS induced through elevation in cytoplasmic  $Ca^{2+}$  (see Example 3, supra), we considered whether the expression of this protein might also determine the extent to which PS is mobilized to cell surfaces under conditions of programmed cell death. As previously reported, the extent to which PS is exposed at cell surfaces when cells are subjected to conditions that ultimately result in their apoptotic death, vary widely between different cell lines, suggesting that the reorganization of plasma membrane phospholipids during programmed cell death is a regulated response inherent to particular cell lines (T. Frey, et al., J. Cytometry 28:253-263, 1997). In particular, both Raji and HL60 have recently been noted to be relatively resistant to

apoptotic induction of cell surface PS (D.L. Bratton, et al., supra, 1997; T. Frey, supra, 1997), consistent with the resistance we observe in the response of these cell lines to a direct elevation of  $[Ca^{2+}]_c$  (See Example 3, above). We therefore compared PS exposure in the B-lymphoblast cell lines Raji (low level PL scramblase expression) and W9 (high level PL scramblase expression) under conditions of serum starvation which has been shown to induce apoptotic death in human B-lymphoblasts (T. Frey, supra, 1997).

Fig. 14 illustrates the role of PL scramblase in PS exposure during apoptosis of B-lymphoblasts. Referring to Fig. 14, PS exposure (measured with Fva light chain) in human B-lymphoblast cell lines during apoptosis induced by one day incubation in reduced serum (1% fetal bovine serum; *solid bars*). Open bars represent identically matched controls maintained continuously in 10% serum. Panel A compares the Raji (low endogenous PL scramblase expression) and W9 (high PL scramblase expression) cell lines. Panel B compares individual Raji cell clones that were stably transfected with either pEGFP-C2 (GFP) or with pEGFP-PL scramblase (GFP-PLS) and then induced to apoptosis under the conditions described for Panel A. Error bars denote mean  $\pm$  SD ( $s=4$ ). Significance of the increase in PS exposure due to serum starvation by paired t-test,  $*p<0.001$  (W9);  $**<0.02$  (GFP-PLS).

As illustrated by Fig. 14 (panel A), when each cell line was incubated under conditions inducing apoptosis, PS exposure observed for W9 was always substantially elevated above that observed for Raji, consistent with both the greater quantity of PL scramblase protein expressed in the W9 cell line and this cell's increased sensitivity to PS mobilization through  $\text{Ca}^{2+}$  ionophore. This increased activity of the PL scramblase pathway in W9 versus Raji was also apparent when apoptosis was induced in these cells by exposure to etoposide (data not shown). Furthermore, we observed that the extent to which PS was mobilized to the surface of Raji cells undergoing apoptosis was increased through prior transfection of these cells to express the GFP-PL scramblase fusion protein (Fig. 14, panel B). These data suggest that the extent to which plasma membrane PS becomes exposed at the cell surface during induced apoptosis of lymphoblasts and other cell lines is directly related to the cellular content of PL scramblase protein.

Expression of PL scramblase promotes intracellular events that result in apoptotic cell death. In order to determine whether the level of expression of PL scramblase alters the capacity of a cell to respond to an external signal to undergo programmed cell death, we used the GFP-PL scramblase-transfected Raji cell lines to monitor fragmentation of nuclear DNA in response to known apoptotic stimuli. In these experiments, the transfected

cells were induced to apoptosis by either culture under serum starvation conditions or by challenge with etoposide. Nuclear degradation was monitored by TUNEL assay (Clontech) and data analyzed as per cent of all  
5 cells induced to DNA fragmentation.

Fig. 15 illustrates transfection with cDNA encoding PL scramblase increases apoptotic response of human B-lymphoma line Raji. Raji cell lines were stably transfected with plasmid to express either GFP-PL  
10 scramblase fusion protein (GFP-PLS; hatched bars) or pGFP vector as control (GFP; solid bars) and analyzed under conditions of induced apoptosis in cell culture. Also shown are data for non-transfected parental cell line Raji (Raji; open bars). Each cell line was incubated  
15 overnight under either serum-starved conditions (0% serum) or in presence of 60  $\mu$ M etoposide (Etoposide). Control refers to identical cells maintained under normal culture conditions of 10% fetal bovine serum. Apoptosis was assessed by degradation of nuclear DNA, as measured  
20 by TUNEL assay (Clontech) and is expressed as percent of total cells induced to undergo nuclear fragmentation. Data of a single experiment, representative of at least five similar experiments so performed.

As shown in Fig. 15, these experiments revealed that  
25 whereas untransfected Raji cells or GFP-vector transfected Raji cell lines were highly resistant to induction of apoptosis, the Raji cell lines expressing the GFP-PL scramblase fusion protein were readily induced



to apoptosis, culminating in fragmentation of nuclear DNA. Consistent with these data, we also observed that the cell lines W9 and Jurkat (expressing high levels of endogenous PL scramblase) were markedly more sensitive to  
5 induction of apoptosis than the cell line Raji (which normal expresses little PL scramblase (data not shown)). In addition to suggesting that the sensitivity of a cell to induction of programmed cell death can be related to the level of expression of PL scramblase, these  
10 experiments establish that a transformed tumor cell line (the human Burkitts lymphoma cell line, Raji) that is normally resistant to apoptosis, can be altered through transfection with cDNA encoding PL scramblase to be sensitive to induction of programmed cell death.

## CLAIMS

We claim:

1. A method for extending the viability of mammalian cells or tissues comprising the step of inhibiting the expression of native PL scramblase within the cell or tissue.

2. The method of claim 1 wherein the inhibition is *in vitro*.

3. The method of claim 1 wherein the inhibition is *in vivo*.

4. The method of claim 1 wherein the mammalian cell or tissue is selected from the group consisting of a blood cell stored for transfusion, a hematopoietic stem cell, an endothelial cell, a pancreatic islet cell, a blood vessel, a skin or other tissue graft, or solid organ for transplantation.

5. The method of claim 1 wherein the inhibition is via a PL scramblase antisense RNA molecule.

6. The method of claim 1 wherein the cell or tissue is a human cell or tissue.

7. The method of claim 1 wherein expression is inhibited via a mutant or truncated form of PL scramblase protein that is inhibitory to endogenous PL scramblase protein.

8. The method of claim 7 wherein the truncated form of the protein is the translation product of an alternatively spliced PL scramblase mRNA.

9. The method of claim 7 wherein the mutant PL scramblase contains one or more non-conservative substitution of residues in the Asp<sup>273</sup>-to-Asp<sup>284</sup> region of PL scramblase polypeptide.

10. The method of claim 1 wherein the function of cell PL Scramblase is inhibited by preventing fatty acylation of the nascent PL scramblase polypeptide.

11. The method of claim 1 wherein the function of cell PL scramblase is inhibited by deacylation of the mature PL scramblase protein.

12. a method of decreasing the viability, metastatic or invasive potential of cancer cells, cancerous tissue, or viral-infected cell by causing increased expression or activity of PL scramblase protein  
5 within the cell or tissue.

13. The method of claim 12 wherein the cancer cell represents a leukemia, lymphoma, carcinoma, adenoma, sarcoma, or other transformed cell type with oncogenic, metastatic, or tumorigenic potential.

14. The method of claim 12 wherein the cell is infected with a human retrovirus, human leukemia virus, human adenovirus.

15. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell is transfected to express an exogenous PL scramblase coding sequence.

16. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell expresses exogenous PL scramblase protein.

17. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell is within a human patient.

18. The method of claim 12 wherein the increased expression of PL scramblase protein is achieved by decreasing expression of alternatively spliced transcripts of PL scramblase within the cancer cell,  
5 cancerous tissue or viral-infected cell.

19. A method of determining the status of a patient's cancer comprising the steps of analyzing the level of PL scramblase RNA or PL scramblase protein within cancer cells and correlating the level with a standard curve.

20. A method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue comprising the step of analyzing the amount of PL scramblase RNA or protein within a patient's cell sample.

21. The methods of claims 19 or 20 wherein the level of PL scramblase RNA is measured by *in situ* hybridization or Northern blotting with PL scramblase cDNA or by the polymerase chain reaction using oligonucleotide primers based on sequence of human PL scramblase cDNA.

22. The method of claims 19 or 20 wherein the level of PL scramblase protein is measured by immunofluorescence, flow cytometry, Western blotting, radioimmunoassay, or by ELISA assay using antibody that specifically binds PL scramblase protein.

23. A method for inducing apoptosis in a cell comprising the step of increasing expression of PL scramblase within the cell.

24. The method of claim 23 wherein the increase in PL scramblase expression is by activating the promoter of an endogenous PL scramblase gene.

25. The method of claim 23 wherein the increase in PL scramblase expression is by transfection of the cell with cDNA encoding PL scramblase.

26. The method of claim 23 wherein the cell is part of a tissue.

27. A method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue comprising the step of analyzing the sequence of a patient's PL scramblase gene, RNA or  
5 protein for the presence or absence of mutations.

28. The method of claim 27 wherein the analysis comprises techniques selected from the group consisting of RFLP, sequencing by RT-PCR, Northern blotting, Western blotting, electrophoretic gels, protease digestion and  
5 other techniques designed to analyze DNA, RNA or protein sequence.

1 cgcggccgcgtcgaccgaaaccaggagccgcgggtgttggcgcaaagggttactcccagac  
61 ccttttccggctgacttctgagaagggttgccgcagcagctgtgcccgcagctctagaggcg  
121 cagaagaggaagccatcgccctggccccggctctctggaccttgtctcgctcgggagcggg  
181 aacagcggcagccagagaactgttttaatcatggacaaacaaaactcacagatgaatgct  
1 M D K Q N S Q M N A  
241 tctcaccgccgaaacaaacttgcaggttgggtatcctcctcagtatccaccgcagcattc  
11 S H P E T N L P V G Y P P Q Y P P T A F  
301 caaggacctccaggatatagtgggtacccctgggccccagggtcagctacccacccccacca  
31 Q G P P G Y S G Y P G P Q V S Y P P P P  
361 gccggccattcagggtcctggccccagctgggtttcctgtcccaaatcagccagtgataat  
51 A G H S G P G P A G F P V P N Q P V Y N  
421 cagccagtatataatcagccaggttggagctgcaggggtacccatggatgccagcgcacag  
71 Q P V Y N Q P V G A A G V P W M P A P Q  
481 cctccattaaactgtccacctggattagaatatattaagtcagatagatcagatactgatt  
91 P P L N C P P G L E Y L S O I D O I L I  
541 catcagcaaatagaacttctgggaagttttaacagggttttgaaactaataacaaatatgaa  
111 H O O I E L L E V L T G F E T N N K Y E  
601 attagaacagctttggacagagggtttactttgcagcgggaagatactgattgctgtacc  
131 I K N S F G Q R V Y F A A E D T D C C T  
661 cgaaattgctgtgggccatctagaccttttaccttgaggattattgataatatgggtcaa  
151 R N C C G P S R P F T\* L R I I D N M G Q  
721 gaagtcataactctggagagaccactaagatgtagcagctgttgttccctgtgctt  
171 E V I T L E R P L R C S S C C C P C C L  
781 caggagatagaaatccaagctcctcctgggtgtaccaatagggttatgttattcagacttgg  
191 Q E I E I Q A P P G V P I G Y V I Q T W  
841 caccatgtctaccaaagtttacaattcaaaatgagaaaagagaggatgtactaaaaata  
211 H P C L P K F T I Q N E K R E D V L K I  
901 agtggteccatgtgttgtgtgcagctgttgtggagatgttgatttttgagattaaatctctt  
231 S G P C V V C S C C G D V D F E I K S L  
961 gatgaacagtggtgtgttggcaaaatttccaagcactggactggaatttttgagagaggca  
251 D E Q C V V G K I S K H W T G I L R E A  
1021 ttacagacgctgataaactttggaatccagttcccttttagaccttgatgtttaaataaaaa  
271 F T D A D N F G I Q F P L D L D V K M K  
1081 gctgtaattgattgggtgcctgtttcctcattgacttcatgttttttgaaagcactggcagc  
291 A V M I G A C F L I D F M F F E S T G S  
1141 caggaacaaaaatcaggagtggtgttagtgattagtgaaagtctcctcaggaaatctgaa  
311 Q E Q K S G V W -  
1201 gtctgtatattgattgagactatctaaactcatacctgtatgaattaagctgtaaggcct  
1261 gtacctctgggtgtatacttttgccttttcaaattatagtttatcttctgtataactgatt  
1321 tataaagggtttttgtacatttttaatactcatttgtcaattttgagaaaaaggacatatga  
1381 gtttttgcatttatataatgaaacttcccttgaaaaactgctttaaaaaaaagtgcacgcg  
1441 gccgc

FIG. 1A

1 tctaaagactcaggaacacaaacctaattgcctcaaagttcagggtgctttttctccctg  
61 acttttagtctagtggagtagtgagcacctatgcctttctgagaggagtctggagagctg  
121 agtcgctgctgggtgctaggattctaggaattcgctcacttggagctgcatgagaaaaga  
181 aaggcttgcaaatggaggctcctcgctcaggaacatacttgccagctgggtatgccccctc  
1 M E A P R S G T Y L P A G Y A P Q  
241 agtatcctccagcagcagtcacaggacctccagagcatactggacgccccacattccaga  
18 Y P P A A V Q G P P E H T G R P T F Q T  
301 ctaactaccaagttccccagtcctggttatccaggacctcaggctagctacacagttctcaa  
38 N Y Q V P Q S G Y P G P Q A S Y T V S T  
361 catctggacatgaagggttatgctgctacacggcttccctattcaaaataatcagactatag  
58 S G H E G Y A A T R L P I Q N N Q T I V  
421 tccctgcaaacactcagtggtgagccagcaccaccacctattctgaactgcccacctgggc  
78 L A N T Q W M P A P P P I L N C P P G L  
481 tagaatacttaaatcagatagatcagcttctgattcatcagcaagttgaacttctagaag  
98 E Y L N Q I D Q L L I H Q Q V E L L E V  
541 tcttaacaggcttttgaacaaataacaaatttgaatcaagaacagcctcgggcagatgg  
118 L T G F E T N N K F E I K N S L G Q M V  
601 tttatgttgagtggaagatactgactgtgtactcgaaattgctgtgaagcgtctagac  
138 Y V A V E D T D C C T R N C C E A S R P  
661 ctttcaccttaagaatcctggatcatctgggccaagaagtcagactctggagcgacctc  
158 F T L R I L D H L G Q E V M T L E R P L  
721 tgagatgcagtagctgctgcttccccctgctgectccaggagatagaaatccaggctcctc  
178 R C S S C C F P C C L Q E I E I Q A P P  
781 cgggggtgccaatagggttatgtgactcagacctggcaccctatgtctgccaagctcactc  
198 G V P I G Y V T Q T W H P C L P K L T L  
841 ttcagaaagacaagagggaatgttctaaaagtagttgggtccatgtgttgcatgcacct  
218 Q N D K R E N V L K V V G P C V A C T C  
901 gctgttcagatattgacttttgagatcaagtcctcttgatgaagtgactagaatttggaaga  
238 C S D I D F E I K S L D E V T R I G K I  
961 tcaccaagcagtggttctgggtgtgtgaaagaggccttcacggattcggataactttggga  
258 T K Q W S G C V K E A F T D S D N F G I  
1021 tccaattccccgctagacctggagggtgaagatgaaagctgtgacgcttgggtgcttgcctcc  
278 Q F P L D L E V K M K A V T L G A C F L  
1081 tcatagattacatgttttttgaaggctgtgagtaggaacagaaatccgacctgcagtagg  
298 I D Y M F F E G C E -  
1141 aatcaatgaaagaggacagagaagatctgaagtctacacaaggagatcatatgattgaga  
1201 gacctggggcttttttgatttcttcattgaaatttctcagaatcaagctgttatacatgaa  
1261 gcatagtatgtaacattttgggttttcaaatggtagtttatcttttacattattgggaatag  
1321 acctggataatttatctttatacacttctaaaaaatatgcaccaaattcaagttaaaaaaa  
1381 aaagacgaagagaagtgatgtttttaaataaaacattttatggaaaagtaagttaaatc  
1441 ataattctgggattttatttttctatcttttgttcaattttaaacccttggtagtgctgatttta  
1501 ttataaaattgtactttactatcaaaccctagtttagttttatttcttacagaaatcctccta  
1561 ttattttgaaattacatattttttgaaagcttttttaaagatactattgcctgggaaattc  
1621 ta

FIG. 1B



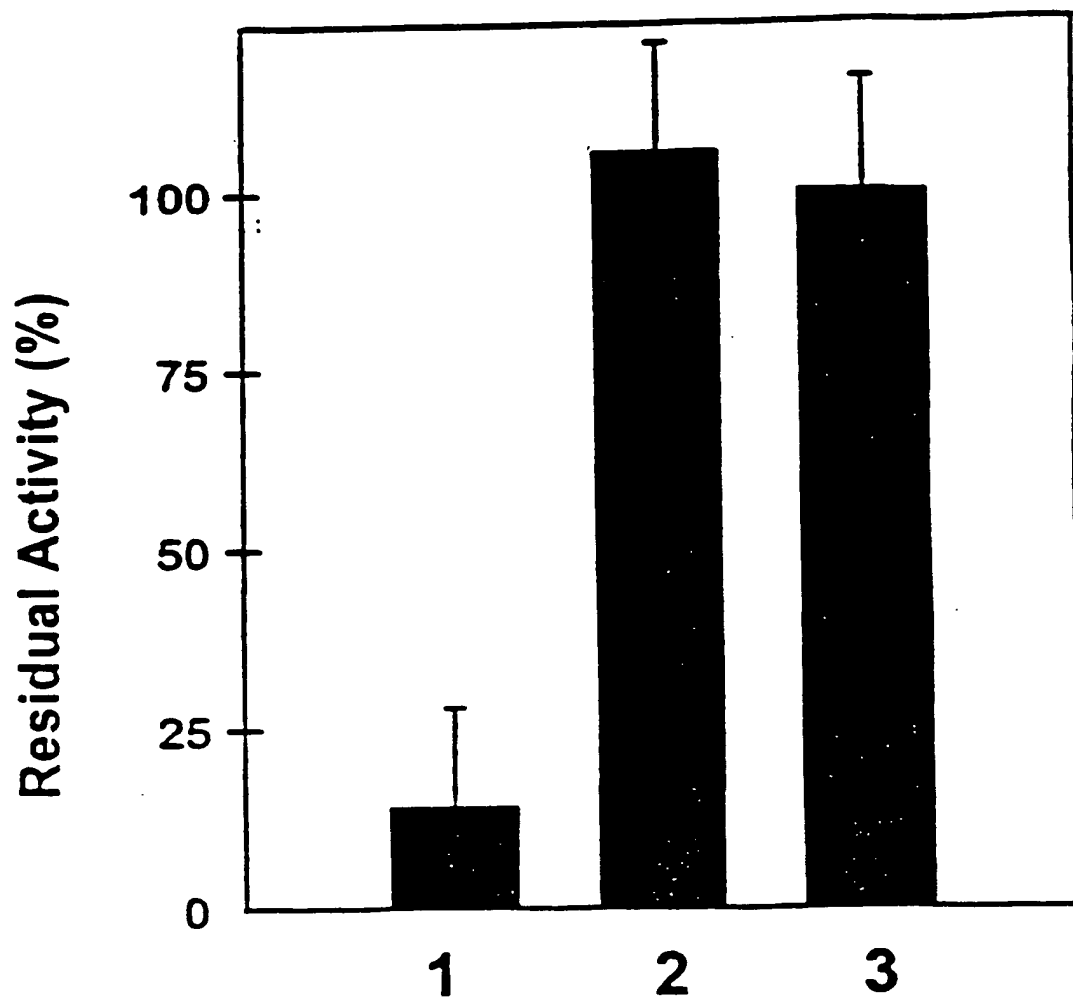


FIG. 2

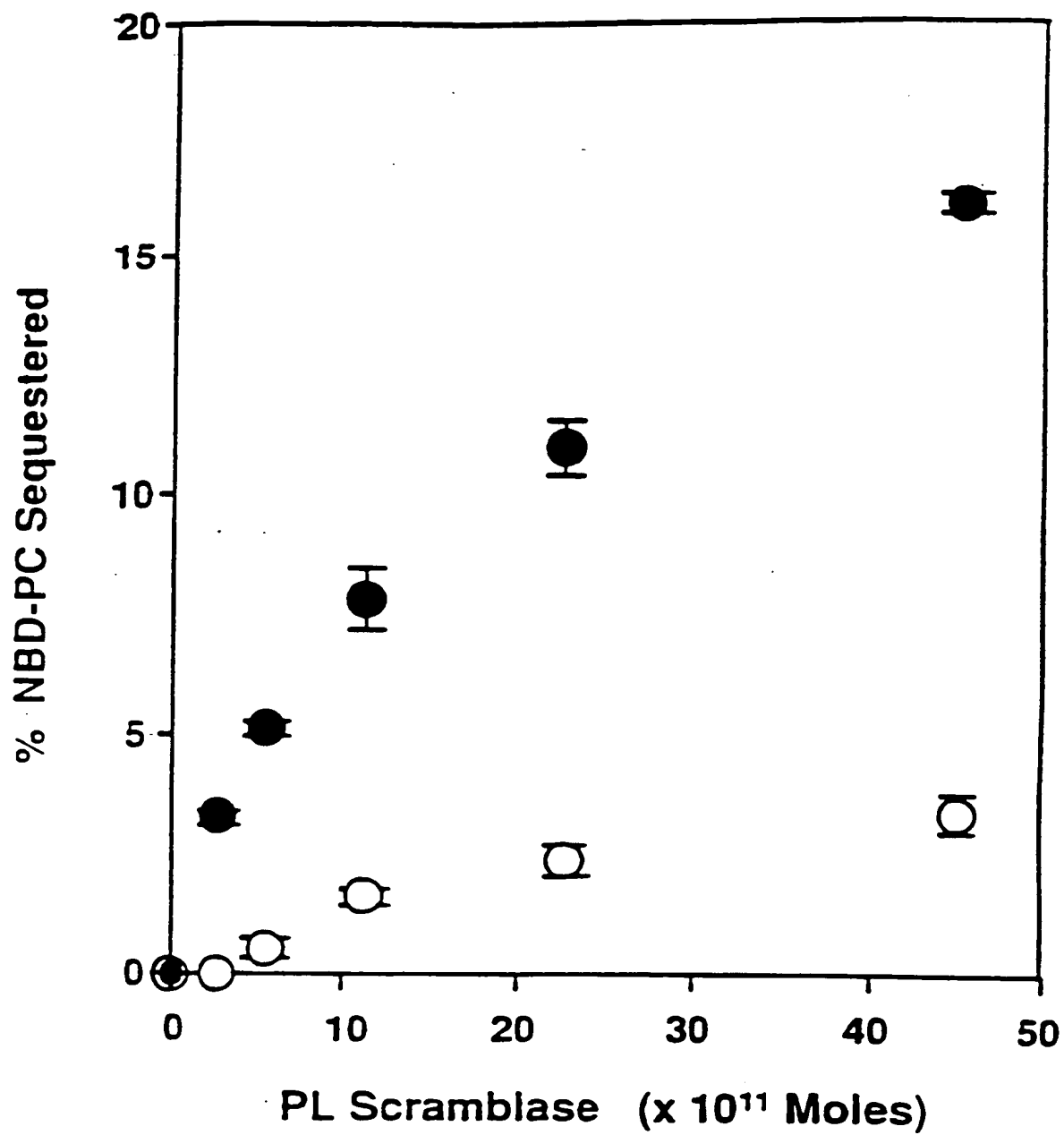


FIG. 3

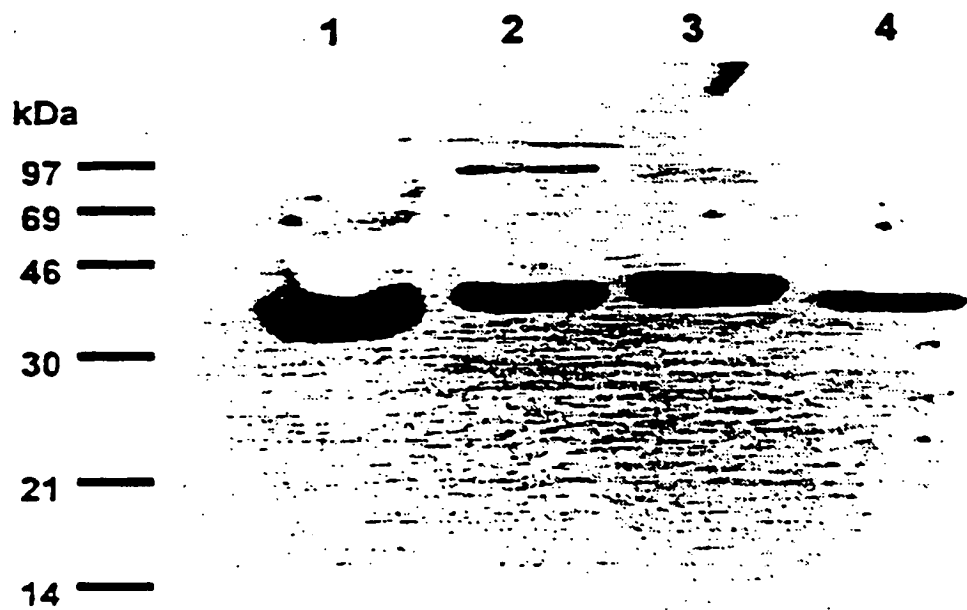
**FIG. 4**

FIG. 5

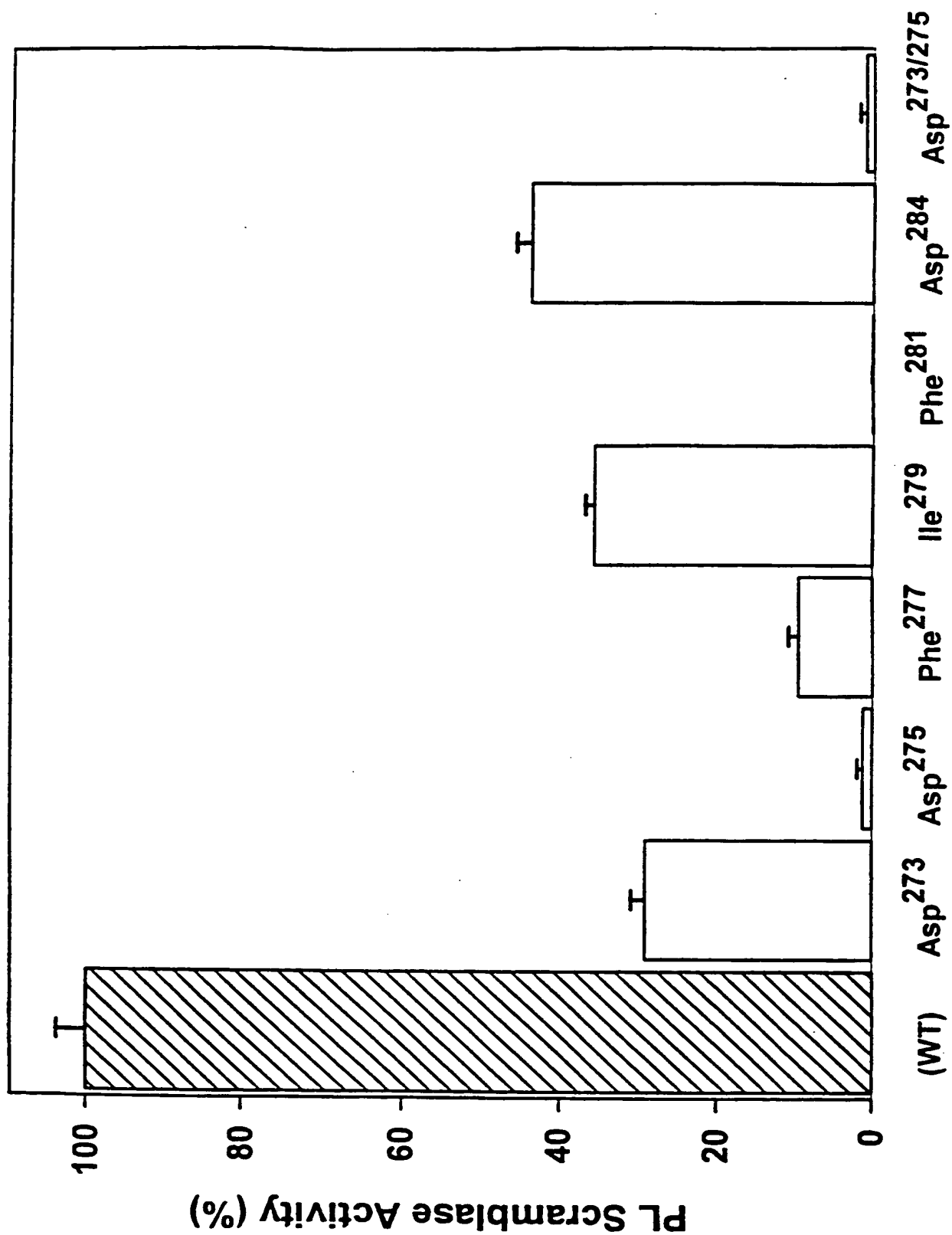


FIG. 6

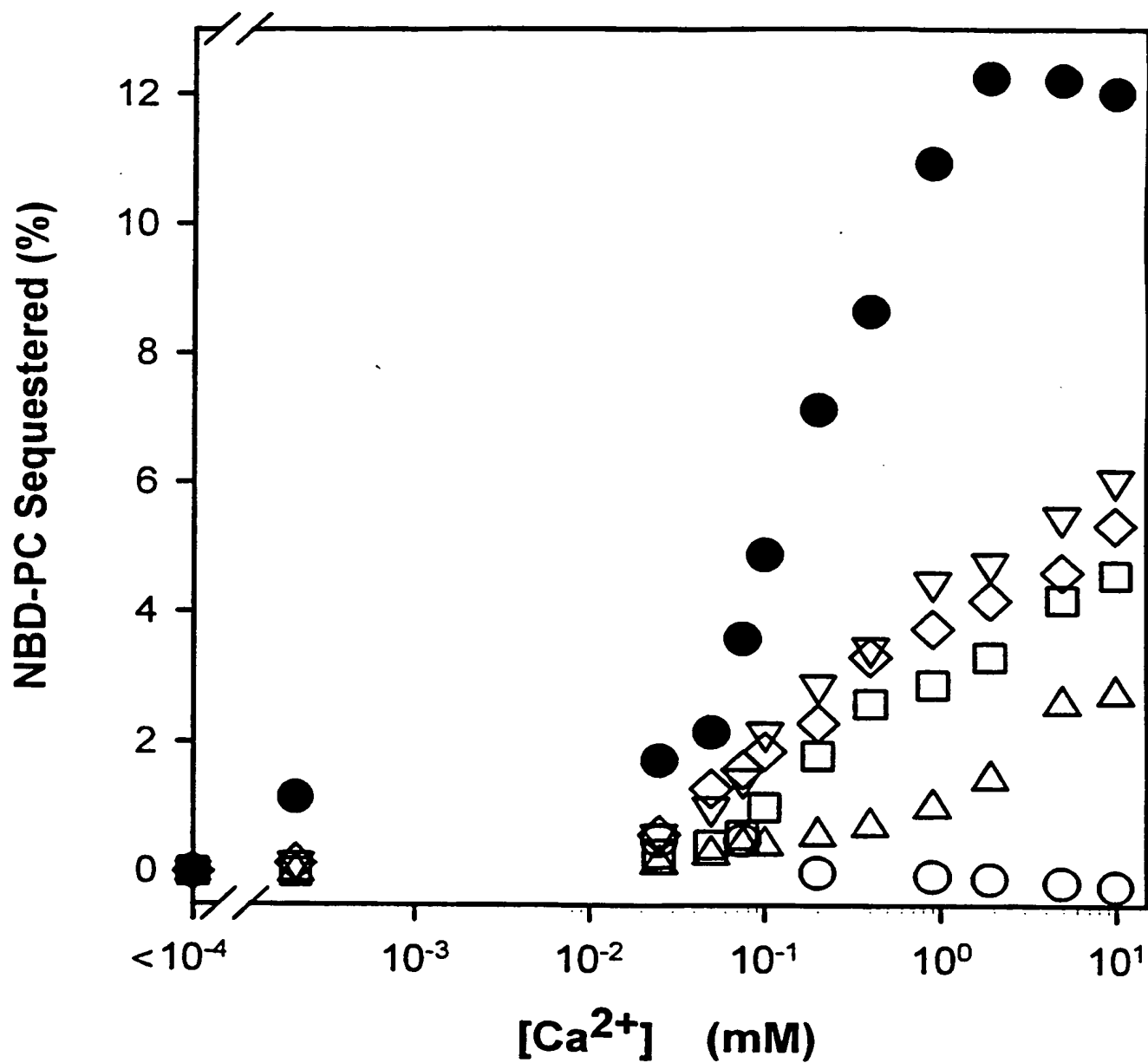


FIG. 7

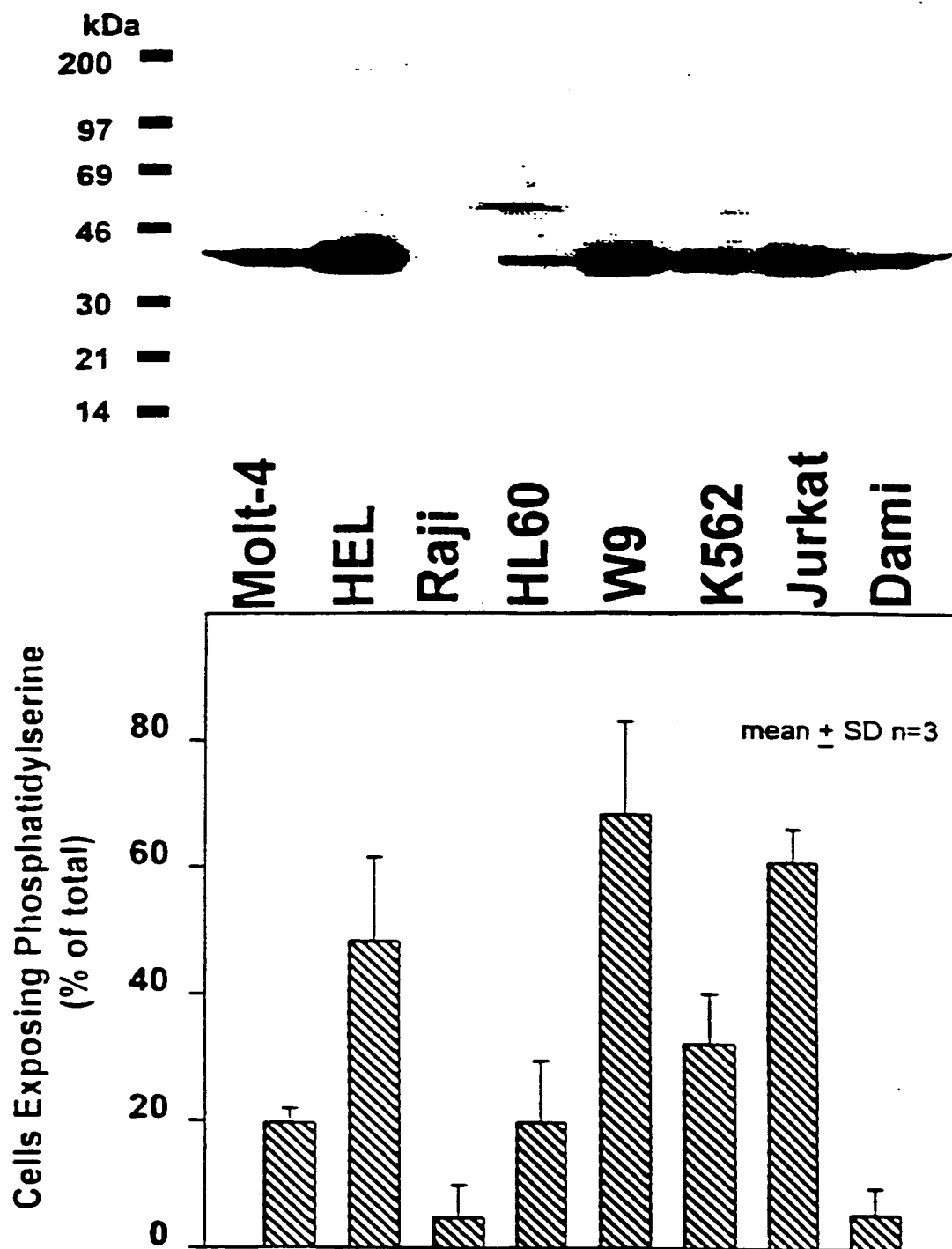
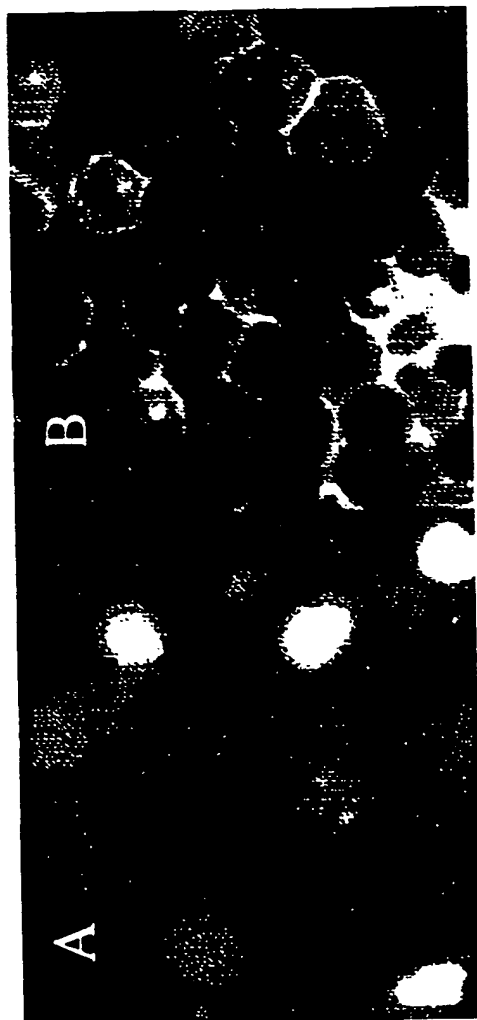


FIG. 8



**FIG. 9**



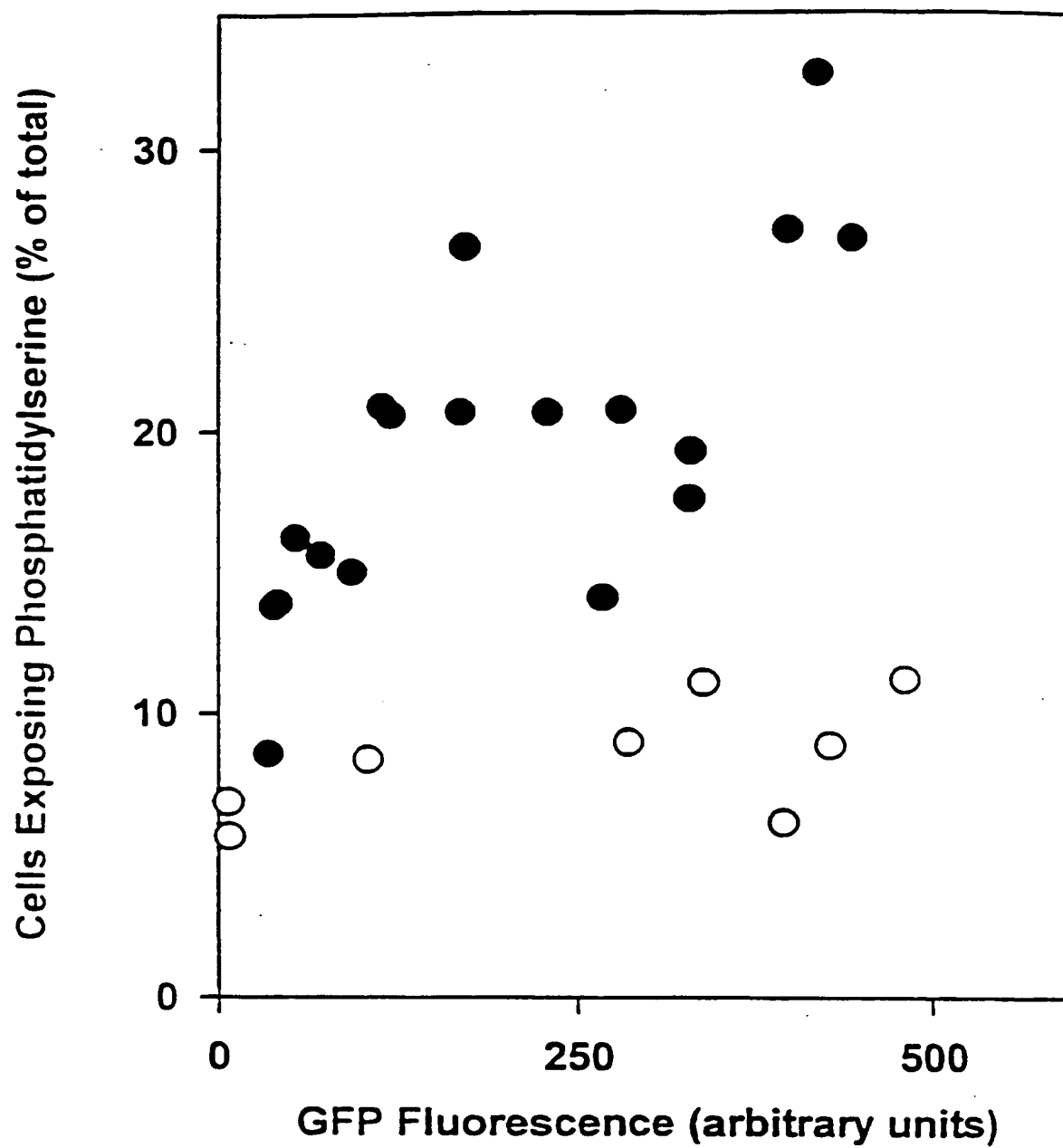


FIG. 10

## Inactivation of PL Scramblase by Thioester Cleavage

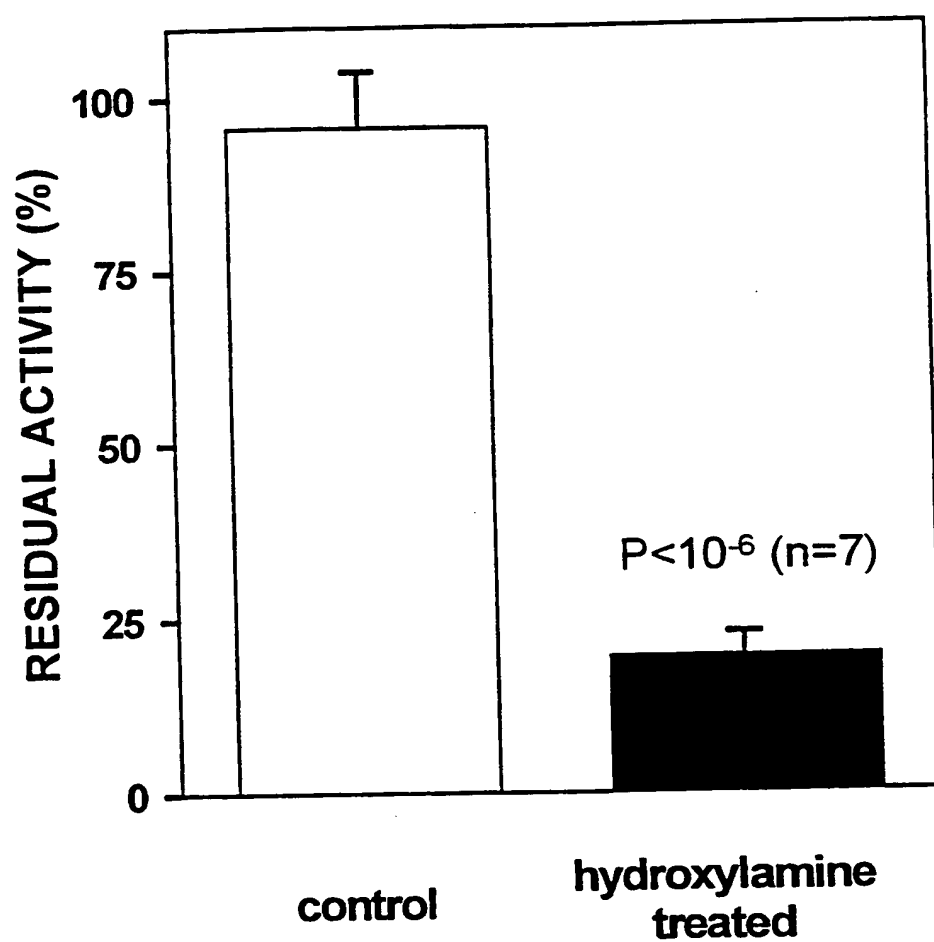
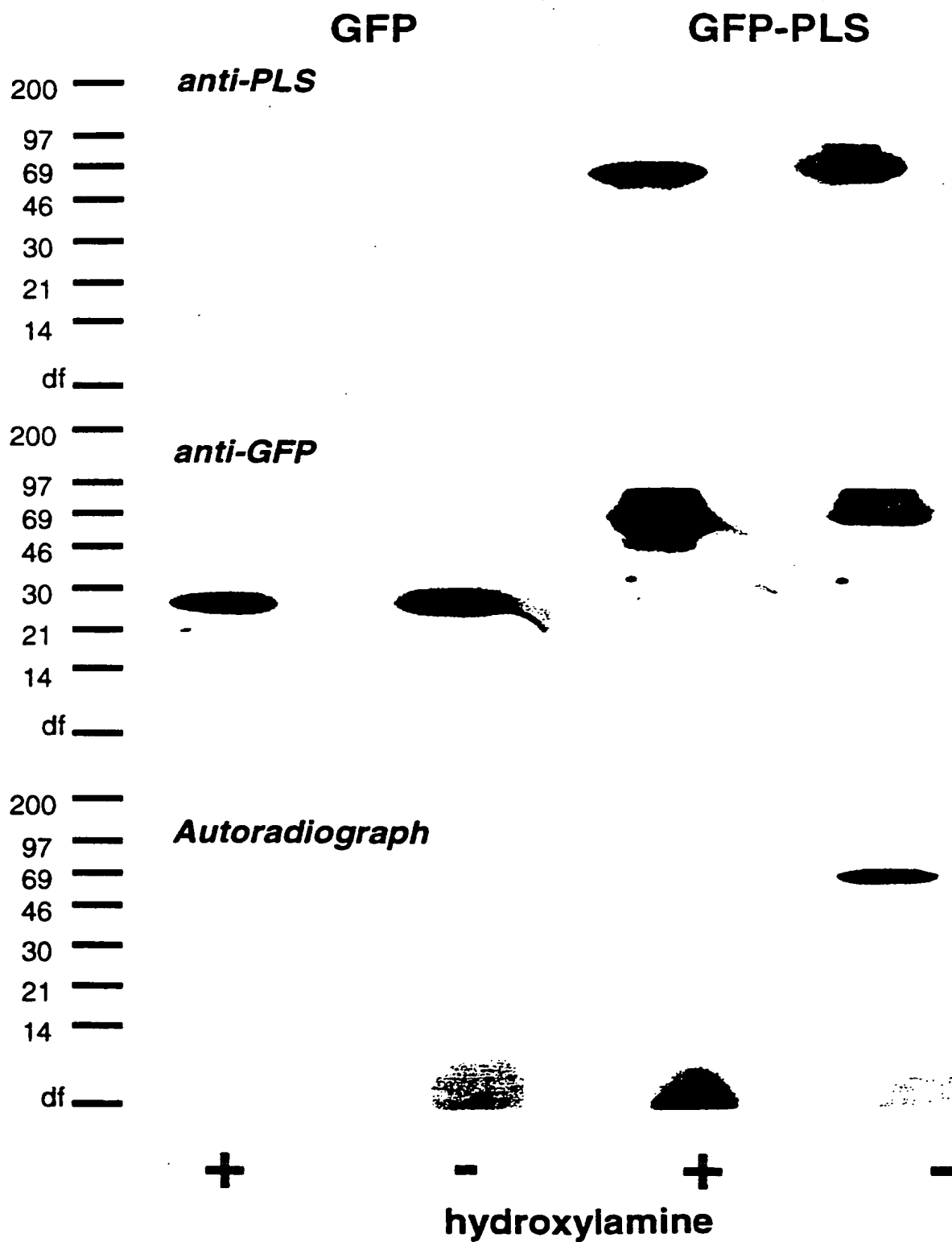


FIG. 11

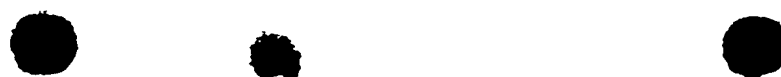
**Metabolic Labeling of PL Scramblase with [<sup>3</sup>H]-Palmitate  
Reveals Covalent Thioester-Linked Fatty Acid**



**FIG. 12**

## TLC Analysis of [ $^3\text{H}$ ]-Fatty Acid From Hydroxylamine-Treated PL Scramblase

SF —



OR —

palmitate

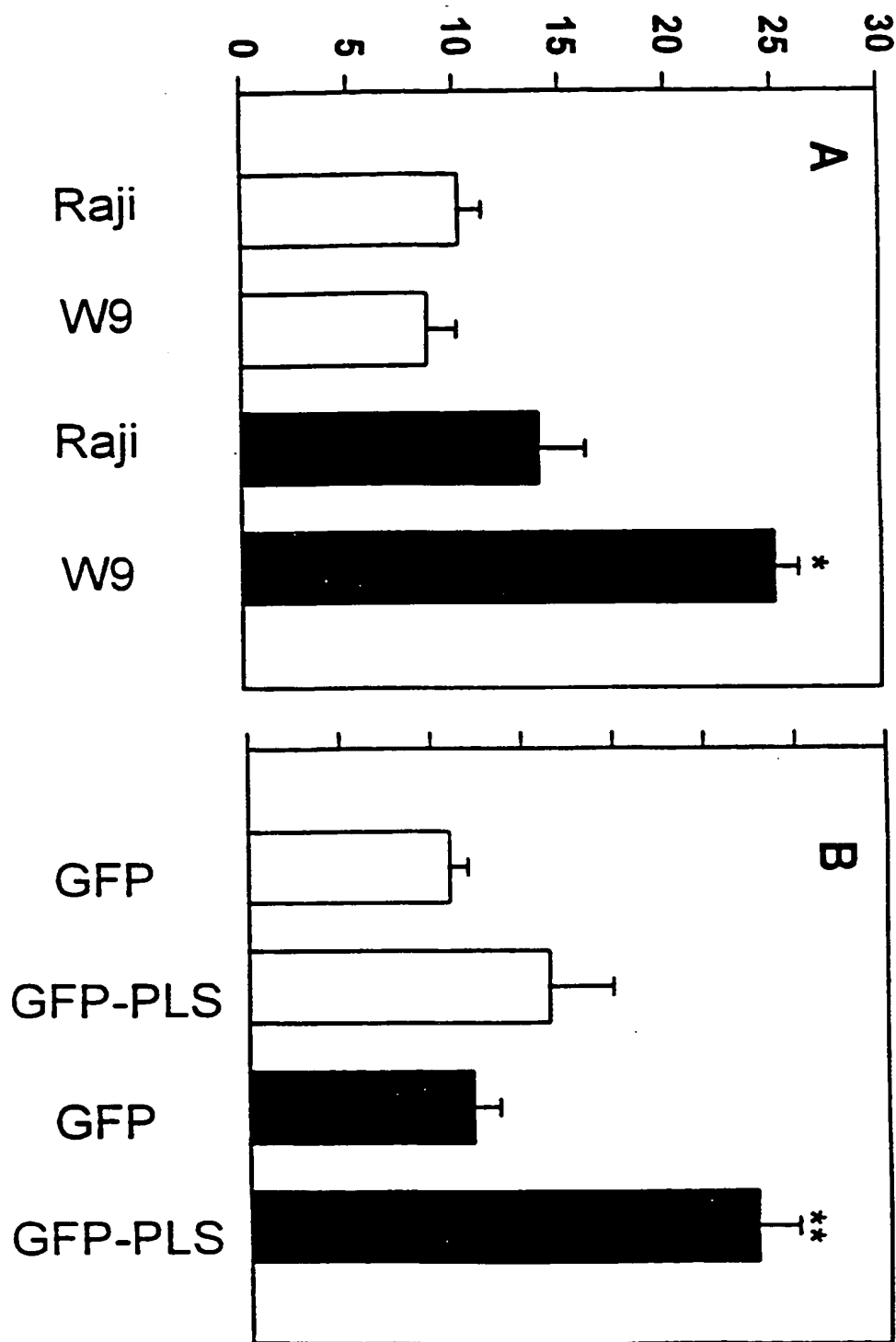
+

-

palmitate

FIG. 13

15 / 16  
**Cells Exposing Phosphatidylserine**  
(% of total)

**FIG. 14**

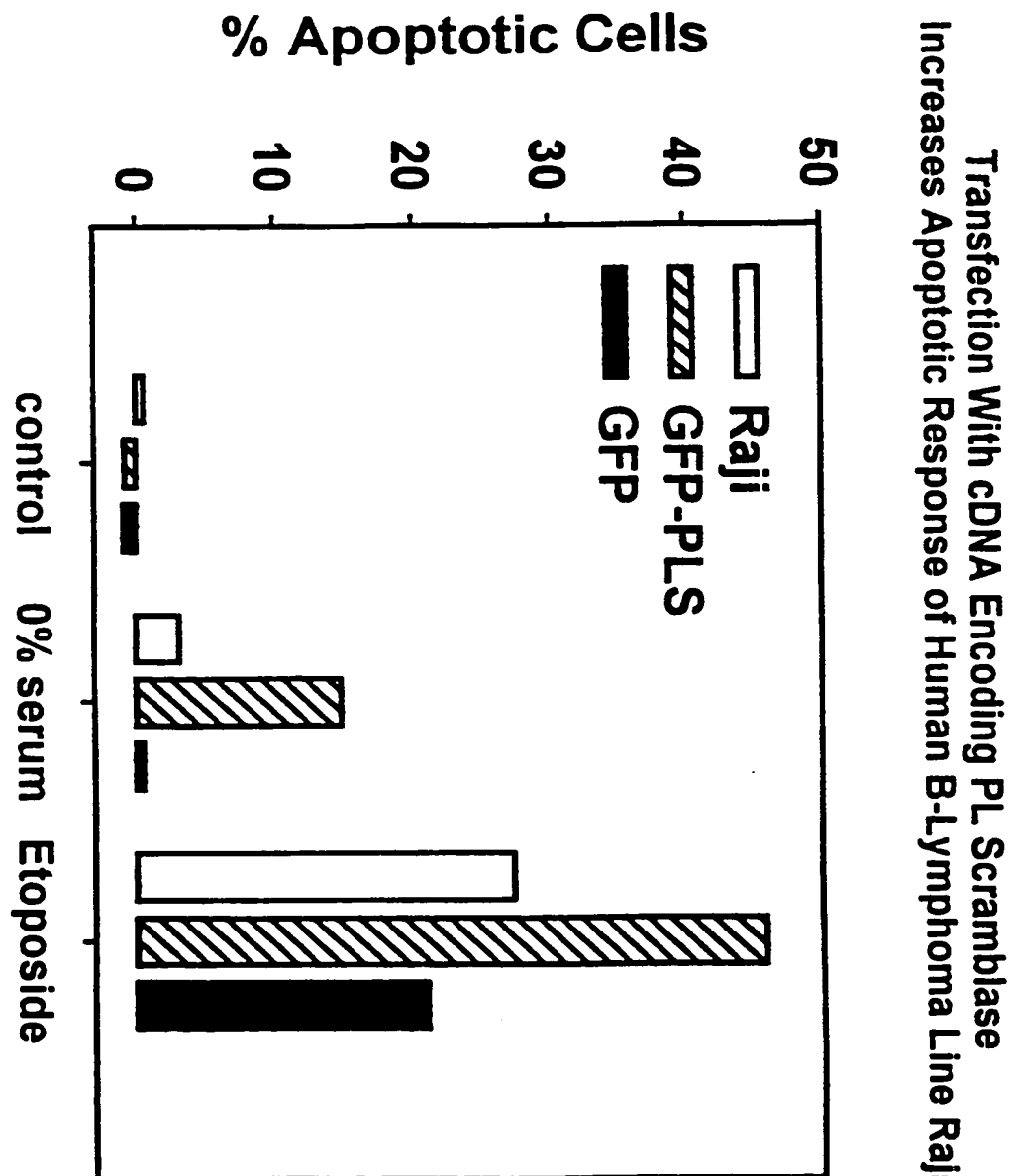


FIG. 15

## SEQUENCE LISTING

<110> Sims, Peter J.  
Zhao, Ji  
Wiedmer, Therese

<120> METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY  
TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO  
CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C12N 15/12, C07K 14/47, C12N 15/11, A61K 38/17, 31/70, C12Q 1/68, G01N 33/68, A61K 48/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 99/36536</b> <b>(43) International Publication Date:</b> 22 July 1999 (22.07.99)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
<b>(21) International Application Number:</b> PCT/US99/01087 <b>(22) International Filing Date:</b> 19 January 1999 (19.01.99) <b>(30) Priority Data:</b> 60/071,950 20 January 1998 (20.01.98) US <b>(71) Applicant (for all designated States except US):</b> BLOOD CENTER RESEARCH FOUNDATION [US/US]; 638 North 18th Street, P.O. Box 2178, Milwaukee, WI 53233 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SIMS, Peter, J. [US/US]; 13386 Caminito Mar Villa, Del Mar, CA 92014 (US). WIEDMER, Therese [US/US]; 13386 Caminito Mar Villa, Del Mar, CA 92014 (US). ZHAO, Ji [US/US]; Apartment 105, 309 North Solano Hills Drive, Solano, CA 92075 (US). <b>(74) Agent:</b> BAKER, Jean, C.; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202-4497 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
<b>(54) Title:</b> METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
<b>(57) Abstract</b> <p>A method for extending the viability of mammalian cells or tissues comprising the step of inhibiting the expression of native PL scramblase within the cell or tissue is disclosed. In another embodiment of the invention, a method of decreasing the viability, metastatic or invasive potential of cancer cells, cancerous tissue, or viral-infected cell by causing increased expression or activity of PL scramblase protein within the cell or tissue is disclosed.</p> <table 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**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY  
TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO  
CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority from U.S. Serial  
No. 60/071,950, filed January 20, 1998.

5 STATEMENT REGARDING FEDERALLY SPONSORED  
RESEARCH OR DEVELOPMENT

United States Government may have commercial rights  
under Grant R01 HL36946 from Heart, Lung, & Blood  
Institute, National Institutes of Health.

BACKGROUND OF THE INVENTION

10 As described in U.S. Serial No. 08/790,186, cell  
surface PS has a role in coagulation, programmed cell  
death and clearance by the reticuloendothelial system.  
U.S. Serial No. 08/790,186 also describes regulation of  
the transmembrane distribution of PS, the role of calcium  
15 in the collapse of phospholipid asymmetry, and the role  
PL translocation in Scott Syndrome.

Programmed Cell Death (Apoptosis)

Programmed cell death or apoptosis is an important  
process that is now recognized to be central to the  
20 selective elimination of mammalian cells during  
embryogenesis, tissue remodeling, the clonal selection of  
immune cells, metamorphosis and hormone-dependent  
atrophy, as well as cell death induced by chemicals or  
irradiation (reviewed by G.M. Cohen, Biochem. J. 326:1-  
25 16, 1997; D.L. Vaux and A Strasser, Proc. Natl. Acad.

Sci. USA 93:2239-2244; D. J. McConkey and S. Orrenius, Biochem Biophys. Res. Commun. 239:357-366, 1997, and references therein). Inappropriate apoptosis (either unregulated excessive or inappropriately low rate) is now  
5 implicated in many human diseases, including Alzheimer's and Huntington's disease, autoimmune disorders, ischemic damage, and several forms of cancer ( M.E. Peter, et al., Proc. Natl. Acad. Sci. USA 94:12736-12737, 1997).

Although the specific pathways leading to cell death may  
10 vary for different cell types and cell stimuli, it is convenient to divide the process into distinct phases. The earliest phase is the stimulus that initiates the apoptotic response. This may be an external signal delivered through surface receptors such as CD95 (Fas) or  
15 tumor necrosis factor receptor, or may originate inside the cell in response to a drug, irradiation or toxin. In the next phase, the signal is detected and transduced to the cell death effector machinery. The third phase of the apoptotic process includes activation of proteases  
20 (including caspases), as well as their positive and negative regulators (including Bcl-2, Bcl-x<sub>L</sub>, and their inhibitors Bax, Bad, and Bak) (J.C. Reed, Nature 387:773-776, 1997; T. Patel, et al., FASEB J. 10:587-597, 1996; G. M. Cohen, supra, 1997). Activation of the "execution  
25 phase" results in morphological changes including cell shrinkage, cell surface exposure of aminophospholipids resulting in the recognition and phagocytosis of apoptotic cells, and plasma membrane blebbing. In the

last phase, chromatin condensation and degradation of DNA is observed. Internucleosomal degradation of DNA into multiples of 180-200 base pair fragments, recognized as "DNA laddering", has long been considered a biochemical  
5 hallmark of apoptosis, although more recent work has shown that the formation of larger DNA fragments, most notably of 50 and 300 kilobases, precedes oligonucleosomal DNA fragmentation in all systems investigated (A.H. Wyllie, Nature 284:555-556, 1980; D.G.  
10 Brown, et al., J. Biol. Chem. 268:3037-3039, 1993).

Phosphatidylserine Exposure in Apoptosis

In recent years it has become clear that externalization of plasma membrane phosphatidylserine (PS) is universally observed in cells undergoing  
15 apoptosis, although the signal transduction pathways leading to surface exposure of PS are poorly understood (B. Verhoven, et al., J. Exp. Med. 182:1597-1601, 1995; S.J. Martin, et al., J. Exp. Med. 182:1545-1556, 1995; D.L. Bratton, et al., J. Biol. Chem. 272:26159-26165,  
20 1997.) PS exposure has been reported to be inhibited by the caspase inhibitor zVAD, implicating this family of "executioner" proteases in this process (S.J. Martin, et al., J. Biol. Chem. 271:28753-28756, 1996). In some cells, inhibitors of calpain have also been reported to  
25 inhibit externalization of PS. However, the molecular link between protease activation and PS exposure remains to be elucidated. As disclosed in US Patent Application Serial No. 08/949,246 and reported in Zhou, et al. (Q.

Zhou, et al., J. Biol. Chem. 272(29):18240-18244, 1997), we recently cloned a protein, PL scramblase, that mediates the  $\text{Ca}^{2+}$ -activated bi-directional transport of phospholipids between the two leaflets of a lipid bilayer membrane. This protein has been shown to transport phospholipids independent of the lipid headgroup, and we now have evidence that PL scramblase is mediating surface exposure of plasma membrane PS in all cells undergoing apoptosis (see below).

#### 10 Role of Ceramide in Apoptosis

In recent years data have accumulated that suggest a major role for the sphingolipid ceramide as a signal transducer in the apoptotic process. It is now evident that in response to a number of stimuli, including tumor necrosis factor, ligand binding or cross-linking of Fas, serum starvation, or  $\gamma$ -irradiation, production of ceramide is observed (Y. Hannun, Nature 274:1855-1859, 1996; R. Kolesnick and Z. Fuks, J. Exp. Med. 181:1949-1952, 1995). Conversely, exogenous addition of ceramide to cells induces apoptosis. Activation of the so-called sphingomyelin cycle results in activation of sphingomyelinase which hydrolyses plasma membrane sphingomyelin to generate cellular ceramide. Ceramide, in turn, has emerged as a candidate intracellular mediator, affecting the activities of several enzymes, including a ceramide-activated protein phosphatase (CAPP), ceramide-activated kinases (CAPK, JNK/SAPKs), and protein kinase C (I. Herr, et al., EMBO J. 16:6200-6208,



1997). In addition to activation of sphingomyelinase, *de novo* synthesis of ceramide in response to an apoptotic stimulus has also been considered. The signal transduction pathways leading to activation of sphingomyelinase remain largely unresolved. It has been noted that cytokine response modifier A (CrmA), a potent inhibitor of caspases, inhibited ceramide formation and tumor necrosis factor-induced cell death, suggesting a role for proteases in the signaling and/or activation phase of apoptosis upstream of ceramide formation (G. Ddaibo, et al., J. Exp. Med. 185:481-490, 1997). As sphingomyelin is normally located primarily in the outer leaflet of the plasma membrane (A.J. Schroit and R.F.A. Zwaal, supra, 1991), it is likely that ceramide generation in the resting cell is limited by the restricted accessibility of sphingomyelin for hydrolysis by the cytoplasmic enzyme, sphingomyelinase, and that the plasma membrane phospholipid rearrangement upon induction of apoptosis results not only in surface exposure of PS, but also movement of sphingomyelin from outer to the inner leaflet of the plasma membrane. As recent evidence from our laboratory suggests that activation PL scramblase is involved in the externalization of PS in apoptotic cells and in the accelerated inward movement of the polar phospholipids phosphatidylcholine and sphingomyelin, we propose that activation of this pathway also provides additional sphingomyelin to the inner cytoplasmic leaflet of the plasma membrane as substrate

for ceramide production. As detailed below, this invention relates to compositions and methods for controlling cell and tissue susceptibility to apoptosis by modifying the transmembrane movement of the plasma membrane phospholipids between inner and outer leaflets.

#### Role of Aminophospholipids in Cell Clearance

In addition to the role that accelerated transbilayer movement of plasma membrane phospholipids may play in ceramide production and in transducing the apoptotic response, the concomitant *de novo* surface exposure of aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) is implicated in the activation of both complement and coagulation systems after tissue injury, and in removal of injured or apoptotic cells by the reticuloendothelial system (A.J. Schroit and R.F.A. Zwaal, Biochim Biophys. Acta 1971:313-329, 1991; V.A. Fadok, et al., J. Immunol. 148:2207-2216, 1996; R.H. Wang, et al., J. Clin. Invest. 92:1326-1335, 1993; D. Pradhan, et al., Mol. Biol. Cell 8:767-778, 1997). Although migration of these phospholipids (PL) from inner to outer plasma membrane leaflets is known to occur as an early event either in programmed cell death or in response to complement-mediated cell injury, and to be related to an elevation of intracellular  $[Ca^{2+}]$  ( $[Ca^{2+}]_i$ ), until recently, little was known about the cellular constituents that participate in this process of reorganizing the topology of the plasma membrane phospholipids.

Relationship of Plasma Membrane Phospholipids to Cancer

Transformed cancer cells exhibit the capacity to proliferate through unregulated mitotic division and to resist the normal cycle of senescence and programmed cell death characteristic of most normal untransformed cells. Additionally, malignant cancer cells *in vivo* exhibit the capacity to evade detection or injury by the body's immune defenses, including cellular killing by cytotoxic T-lymphocytes, humoral killing by antibody and complement, and removal by macrophages and other phagocytic cells of the reticuloendothelial system. Whereas there is now little information as to the specific role played by the plasma membrane phospholipids in the oncogenic and malignant potential of a cell, the potential importance of the transbilayer transport of phospholipid between leaflets of the plasma membrane to the growth behavior and senescence of a cell is suggested by evidence that (1) sphingomyelin-derived ceramide participates in the intracellular signaling that results in apoptosis (*supra*) (2) early events in both apoptosis and immune injury include the cell surface exposure of the aminophospholipids PS and PE, (*supra*) and (3) cell surface PE has been shown to promote activation of the complement system including generation of the opsin C3b and the cytolytic complex, C5b-9, whereas cell surface PS has been shown to directly promote clearance through the reticuloendothelial system, by promoting cytoadhesion and phagocytosis by tissue macrophages. (R.H. Wang, *et al.*,

J. Clin. Invest. 92:1326-1335, 1993; V.A. Fadok, et al.,  
J. Immunol. 148:2207-2216, 1992; R.F.A. Zwaal and A.J.  
Schroit, supra, 1997.) This implies that conditions that  
induce collapse of the normal plasma membrane  
5 phospholipid asymmetry, i.e., that promote the ingress of  
sphingomyelin from outer to inner plasma membrane leaflet  
and the egress of PS and PE from inner to outer leaflet,  
would serve to accelerate apoptosis (through generation  
of ceramide) and to accelerate cell removal through the  
10 reticuloendothelial system (through combined effects of  
cell surface PS and PE). Consistent with this premise,  
Der, et al. (Der, et al., Proc. Natl. Acad. Sci. USA  
95:15623-15628, 1998) reported that treatment with  
interferons markedly elevates expression of certain  
15 apoptosis-related genes, including PL scramblase. Der,  
et al. propose that the anti-tumor activity of  
interferons *in vivo* may relate to increased expression of  
PL scramblase in tumor cells containing interferon  
receptors, thereby promoting movement of PS to the cell  
20 surface and accelerating clearance through the  
reticuloendothelial system.

On the other hand, conditions that restrict or  
prevent this transbilayer movement of plasma membrane  
phospholipids would protect from cell death and promote  
25 the perpetual survival of the cell *in vivo*.

Identity of a protein mediating bidirectional transbilayer movement of plasma membrane phospholipids (PL Scramblase)

Bassé, et al. and Stout, et al. recently reported  
5 the purification and preliminary characterization of an  
integral RBC membrane protein that, when reconstituted in  
liposomes, mediates a  $\text{Ca}^{2+}$ -dependent transbilayer movement  
of PL mimicking plasma membrane PL reorganization evoked  
upon elevation of  $[\text{Ca}^{2+}]_c$  (F. Bassé, et al., J. Biol.  
10 Chem. 271:17205-17210, 1996; J.G. Stout, et al., J. Clin.  
Invest. 99:2232-2238, 1997). Evidence that a protein of  
similar function must also be present in platelets was  
recently reported by Comfurius, et al. (P. Comfurius, et  
al., Biochemistry 35:7631-7634, 1996).

15 The molecular identity of this PL scramblase protein  
including cDNA and deduced protein sequence of the full-  
length polypeptide was disclosed in US Patent Application  
Serial No. 08/949,246 and reported in Zhou, et al. (Q.  
Zhou, et al., J. Biol. Chem. 272(29):18240-18244, 1997).  
20 This report also provides evidence that this same protein  
mediates plasma membrane PL scramblase function in a wide  
variety of human cells and tissues. Furthermore, this  
report discloses cell-to-cell differences in the level of  
expression of mRNA encoding PL scramblase, implying  
25 tissue-specific regulation of gene expression.

Analysis of the cDNA-derived protein sequence  
revealed a strongly-preferred ( $p < 0.01$ ) inside-to-outside  
orientation of the predicted 19 residue transmembrane  
helix, consistent with a type II plasma membrane protein.

Most of the polypeptide (residues 1-290) thereby extends from the cytoplasmic membrane leaflet, leaving a short exoplasmic tail (residues 310-318) (Q. Zhou, et al., supra, 1997). The predicted orientation of this protein is consistent with the anticipated topology of PL scramblase in the erythrocyte membrane, where lipid-mobilizing function is responsive to  $[Ca^{2+}]$  only at the endofacial surface of the membrane (Q. Zhou, et al., supra, 1997; P. Williamson, et al., Biochemistry 31:6355-6360, 1992; E.F. Smeets, et al., Biochim. Biophys. Acta BioMembr. 1195:281-286, 1994; F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997; P. Williamson, et al., Biochemistry 34:10448-10455, 1995; D.L. Bratton, J. Biol. Chem. 269:22517-22523, 1994).

Consistent with the divalent ion-dependence of its PL-mobilizing activity, Zhou, et al. identified a conserved EF-hand-like motif in PL scramblase that is required for the  $Ca^{2+}$ -accelerated transbilayer movement of membrane phospholipids (Zhou, et al., Biochemistry 37:2356-2360, 1998), and PL scramblase was shown to undergo a prominent conformational change upon binding  $Ca^{2+}$  (Stout, et al., Biochemistry 37:14860-14866, 1998). Human PL scramblase was shown to be thiol-esterified with palmitate at cytoplasmic cysteinyl residue(s) and evidence provided that the normal PL-mobilizing activity of this protein is dependent upon such post-translational modification of the polypeptide with fatty acid (Zhao, et al., Biochemistry 37:6361-6366, 1998).

In addition to platelet and red blood cell, PL scramblase activity has been observed in many other cells, and this  $\text{Ca}^{2+}$ -induced response is thought to be central to the rapid movement of PS and

5 phosphatidylethanolamine from inner plasma membrane leaflet to the surface of perturbed endothelium, and a variety of injured and apoptotic cells (R.F.A. Zwaal and A.J. Schroit, supra, 1997). The resulting exposure of PS at the cell surface is thought to play a key role in

10 removal of such cells by the reticuloendothelial system, in addition to activation of both the plasma complement and coagulation systems (R.H. Wang, et al., supra, 1993; V.A. Fadok, et al., supra, 1992; R.F.A. Zwaal and A.J. Schroit, supra, 1997; D. Pradhan, et al., supra, 1997).

15 Whereas the molecular mechanism(s) in each circumstance remains unresolved, evidence for a specific platelet membrane protein functioning to accelerate migration of PL between membrane leaflets at increased cytosolic  $[\text{Ca}^{2+}]$  has been reported (P. Comfurius, et al., supra, 1996;

20 Zhou, et al., supra, 1997), similar to the proposed role of PL scramblase in red blood cells (F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997). It was thus of interest to determine whether mRNA for this protein is expressed in nucleated cells where PL

25 scramblase-like activity has been observed.

Northern blotting with PL scramblase cDNA revealed transcripts of ~1.6 and ~2.6 kb in all tissues and cell lines tested (Q. Zhou, et al., supra, 1997). Some

tissue-to-tissue and cell line variability in the relative abundance of these two transcripts is apparent, the significance of which remains to be determined. Also notable was markedly reduced expression in HL-60 and the lymphoma lines Raji and MOLT-4 whereas abundant message was detected in spleen, thymus, and peripheral leukocytes. In addition to the transformed cell lines shown, mRNA for PL scramblase was also confirmed in human umbilical vein endothelial cells. Whereas these data imply that the same protein identified as mediating accelerated transbilayer flip-flop of the erythrocyte membrane PL also plays a similar role in the plasma membrane of platelets, leukocytes and other cells, actual confirmation for this role of PL scramblase awaits analysis of a cell line that is selectively deficient in this protein (Q. Zhou, et al., supra, 1997). In Scott syndrome, a bleeding disorder related to an inherited deficiency of plasma membrane PL scramblase function, erythrocytes deficient in PL scramblase activity were found to contain normal amounts of the PL scramblase protein (J.G. Stout, et al., supra, 1997). Furthermore, despite the apparent deficiency in Scott syndrome cells of endogenous PL scramblase function, when PL scramblase protein from these cells was purified and reconstituted in proteoliposomes containing exogenous PL, it exhibited normal  $\text{Ca}^{2+}$ -dependent PL-mobilizing activity (J.G. Stout, et al., supra, 1997). Consistent with these results, analysis of B lymphoblasts from a patient with Scott



syndrome by Northern blot and RT-PCR revealed normal amounts of RNA and cDNA of PL scramblase, respectively, and cDNA sequence was identical to that previously reported for PL scramblase (Q. Zhou et al., Blood 5 92:1707-1712, 1998). These results suggest that in addition to the known regulation by intracellular  $[Ca^{2+}]$ , the activity of PL scramblase in the plasma membrane is regulated by other as yet unidentified membrane or cytoplasmic component(s).

10 Relationship of PL Scramblase to Cancer

Kasukabe et al., 1997 (T. Kasukabe, et al., Blood 89:2975-2985, 1997) disclose a gene designated NOR1 that is down-regulated in transformed murine monocytes relative to amount of mRNA expressed in normal mouse 15 cells and a 5'-truncated form of this same transcript (designated TRA1) that is over-expressed in leukemogenic mouse monocytic cell lines but not found in the non-leukemogenic monocytic cell lines. They suggest that the Tra1 gene product is associated with the leukemogenesis 20 of monocytic Mm cell lines.

Analysis of the open reading frame predicted by the NOR1 and TRA1 cDNA sequences reveals near-identity of protein sequence with mouse or human PL scramblase in the overlapping portions of each polypeptide. The apparent 25 identity of the full-length NOR1 gene product as the mouse homologue of human PL scramblase was recently confirmed on the basis of cDNA cloning and sequence analysis (T. Kasukabe, et al., Biochem. Biophys. Res.

Commun. 249:449-55, 1998.) As will be detailed below, the present invention relates to our discovery that the level of expression of PL scramblase influences the capacity of cells to resist complement injury or to succumb to stimuli that induce programmed cell death. The present invention also relates to the role of PL scramblase in promoting clearance of injured or apoptotic cells by the phagocytic cells of the reticuloendothelial system.

10        Needed in the art is a method for modulating the activity or level of phospholipid scramblase within a cell, organ or tissue in which one wishes either to reduce the potential for cell death and resulting clearance of beneficial cells by the scavenger  
15        macrophages of the reticuloendothelial system (by decreasing cellular PL scramblase expression or activity) or to promote cell death and cell clearance from the body of undesirable cells (by increasing cellular PL  
20        scramblase expression or activity).

20        BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

      Fig. 1A is a comparison of the cDNA and deduced amino acid sequence of human PL scramblase (SEQ ID NOs:1 and 2).

      Fig. 1B is a comparison of the cDNA and deduced  
25        amino acid sequence of murine PL scramblase (SEQ ID NOs:3 and 4).

Fig. 2 is a bar graph illustrating immunoprecipitation of erythrocyte PL scramblase.

Fig. 3 is a graph of an activity assay of recombinant PL scramblase.

5 Fig. 4 is an immunoblot of PL scramblase in human erythrocytes and platelets.

Fig. 5 is a comparison of protein sequences of mouse and human PL scramblase (SEQ ID NOs:2 and 4).

10 Fig. 6 is a bar graph of PL scramblase activity as a function of mutational analysis of a putative EF hand loop motif contained in human PL scramblase.

Fig. 7 graphs the  $\text{Ca}^{2+}$  dependence of mutant human PL scramblase with amino acid substitutions in putative  $\text{Ca}^{2+}$  binding site.

15 Fig. 8 is a Western blot analysis of PL scramblase protein and corresponding functional assay of PL scramblase activity in various human cell lines.

20 Fig. 9A and B are fluorescence micrographs of GFP-PL scramblase in transformed Raji cells. Fig. 9A depicts fluorescence of cells expressing GFP; Fig. 9B depicts cells transfected with pEGFP-C2-PL scramblase plasmid and expressing GFP-PL scramblase fusion protein.

25 Fig. 10 is a graph showing that the level of expression of PL scramblase determines plasma membrane sensitivity to intracellular calcium.

Fig. 11 is a bar graph illustrating inactivation of PL scramblase by hydroxylamine.

Fig. 12 is analysis of metabolically-labeled PL scramblase by SDS-PAGE revealing covalent incorporation of thioester-linked fatty acid.

Fig. 13 is analysis by thin layer chromatography demonstrating that PL scramblase contains covalent palmitic acid.

Fig. 14A and B is a bar graph illustrating the role of PL scramblase in PS exposure during apoptosis of B-lymphocytes. Fig. 14A compares Raji and W9 cell lines. Fig. 14B compares individual Raji cell clones that were stably transfected with either pEGFP-C2 or with pEGFP-PL scramblase and then induced to apoptosis.

Fig. 15 is a bar graph illustrating the percent apoptotic cells for Raji cells, Raji cells transfected with GFP-PL scramblase fusion protein or GFP vector as a control.

#### DESCRIPTION OF THE INVENTION

The present invention relates to the creation and use of reagents and methods to either inhibit or promote cell injury, cell death, and cell clearance from the body. These reagents and methods rely on the properties of phospholipid scramblase (PL scramblase), a protein that mediates  $\text{Ca}^{2+}$ -dependent transbilayer movement of plasma membrane phospholipids that are involved in the activation of the plasma complement and coagulation cascades and are substrates for cellular enzyme cascades that mediate programmed cell death.

In one embodiment, the present invention is a method for decreasing the viability of cancer cells or virus-infected cells comprising the step of increasing the amount or activity of PL scramblase in the plasma membrane of the cancer cell, cancerous tissue, or virus-infected cell, thereby promoting the movement of plasma membrane phospholipids between inner and outer plasma membrane leaflets. Preferably, the increase in PL scramblase is accomplished by activating the promoter of the endogenous PL scramblase gene or by delivering cDNA encoding PL scramblase, provided in a suitable targeting vector to the cell or tissue. In a preferred embodiment, cDNA encoding PL scramblase is delivered to the cell or tissue in a targeting vector, where the vector is a retrovirus or adenovirus vector containing the PL scramblase cDNA.

The feasibility of this strategy is exemplified by a recent report by Der, et al. (supra, 1998) that treatment of a human fibrosarcoma cell line with interferons markedly elevates expression of a number of apoptosis-related genes, including PL scramblase. Der, et al. hypothesize that the anti-tumor activity of interferons in vivo may relate to increased expression of PL scramblase in tumor cells containing interferon receptors, thereby promoting movement of PS to the cell surface and accelerating clearance through the reticuloendothelial system.

In another embodiment, the present invention is a method for inducing programmed cell death (apoptosis) of a cell, preferably a mammalian cell, comprising the step of increasing the level of expression of plasma membrane PL scramblase, thereby increasing the movement of plasma membrane phospholipids between membrane leaflets, resulting in the movement of the phospholipid sphingomyelin from outer membrane leaflet to inner leaflet and the increase in production of ceramide from sphingomyelin through the action of the cytoplasmic enzyme sphingomyelinase. The resulting ceramide is known to induce activation of a cascade of cellular enzymes that ultimately lead to apoptotic cell death.

In another embodiment, the present invention is a method for promoting clearance of a cell from the body by phagocytic uptake into the reticuloendothelial system comprising the step of increasing the level of expression of plasma membrane PL scramblase, thereby increasing cell surface exposure of phosphatidylserine and phosphatidylethanolamine through the movement of these aminophospholipids from inner plasma membrane leaflet to outer plasma membrane leaflet. Cell-surface-exposed phosphatidylserine is known to directly promote adhesion and phagocytosis by macrophages and other cells of the reticuloendothelial system whereas phosphatidylethanolamine promotes cell adhesion and phagocytosis by macrophages and other cells of the reticuloendothelial system by promoting complement

activation, which opsonizes the cell surface with complement C3b.

In another embodiment, the present invention is a method of promoting the viability of a mammalian cell, both in *in vitro* cell culture and *in vivo*, comprising the step of decreasing the activity of the plasma membrane PL scramblase, thereby inhibiting movement of plasma membrane phospholipids between inner and outer membrane leaflets. The decrease in plasma membrane PL scramblase is accomplished by either decreasing expression of the endogenous PL scramblase gene or by delivering to the cell an inhibitor of the PL scramblase protein. In one embodiment, the expression of PL scramblase is inhibited by delivering to the cell antisense oligonucleotides or antisense cDNA to PL scramblase RNA. In another embodiment, a defective form of the PL scramblase protein is expressed in the cell and that mutant PL scramblase inhibits the endogenous PL scramblase. In the preferred embodiment, endogenous PL scramblase is inactivated by deacylating essential fatty acids from the protein that are required for normal PL scramblase function in the plasma membrane. By decreasing the activity of plasma membrane PL scramblase, the cell is protected due to decreased hydrolysis of plasma membrane sphingomyelin to ceramide (mediated by cellular sphingomyelinase), as well as by decreased exposure of the procoagulant and complement-activating aminophospholipids

phosphatidylserine and phosphatidylethanolamine at the cell surface.

In another embodiment, the present invention is a method of preventing the phagocytic removal and clearance  
5 of a transfused or transplanted cell by preventing surface exposure of plasma membrane phosphatidylserine by delivering to the cell a mutant phospholipid scramblase that inhibits the endogenous PL scramblase. This scramblase is preferably mutated at a site of post-  
10 translational modification, most preferably the site is selected from the group consisting of Asp<sup>273</sup>-Asp<sup>284</sup>, Thr<sup>161</sup> and conserved cysteines of human PL scramblase or the corresponding conserved residues in mouse or other mammalian PL scramblase.

15 In one embodiment, a gene construct encoding a mutant phospholipid scramblase is delivered to the cell. In an alternative embodiment, the mutant protein itself is delivered.

In another embodiment the present invention is a  
20 method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue. This method may comprise the step of analyzing the amount of PL scramblase RNA or protein within a patient's cell sample. We predict that the presence of a comparatively  
25 low amount of PL scramblase RNA or protein will indicate the presence of cancerous tissue. The level of PL scramblase RNA may be measured by *in situ* hybridization or Northern blotting with PL scramblase cDNA or by



polymerase chain reaction using oligonucleotide primers based on sequence of human PL scramblase RNA.

Alternatively, the method may comprise the step of analyzing the sequence of a patient's PL scramblase gene,  
5 RNA or protein for the presence or absence of mutations. This analysis may preferably be selected from the group consisting of restriction fragment length polymorphism, sequencing by RT-PCR, Northern blotting, Western blotting, electrophoretic gels, protease digestion, and  
10 other techniques designed to analyze RNA, DNA or protein sequence.

In a preferred embodiment, the present invention involves the use of a preparation of a plasma membrane phospholipid scramblase ("PL scramblase"). It is now  
15 known that PL scramblases isolated from various species have sequence variations. By "PL scramblase" we include these variants as well as the proteins as described in U.S. Serial No. 08/790,186, Serial No. 08/949,246, Zhao, et al. (J. Biol. Chem. 272(29):18240-18244, 1997), and  
20 Kasukabe, et al., (Biochem. Biophys. Res. Comm. 249:449-55, 1998) and genes or cDNAs encoding these proteins.

Preferably, the protein is approximately 35-37 kD as measured on a 12.5% SDS polyacrylamide gel under reducing conditions. In a most preferred form of this invention,  
25 the preparation is a human or a mouse PL scramblase.

In one preferred embodiment of the present invention, the PL scramblase comprises SEQ ID NO:2, representing human PL scramblase, possibly with conservative or functionally equivalent substitutions.

5 In the most preferred embodiment of the present invention, the PL scramblase has been modified by the action of mammalian cellular enzymes to covalently incorporate phosphorus at one or more Thr, Ser, or Tyr residues or a fatty acid, preferably palmitate, at a  
10 cysteine residue.

The present invention may also use a murine cell protein, wherein the protein is a plasma membrane phospholipid scramblase, preferably wherein the protein is approximately 35 kD as measured on a 12.5%  
15 SDSpolyacrylamide gel under reducing conditions.

In another preferred embodiment of the present invention, the murine PL scramblase has been modified by the action of mammalian cellular enzymes to covalently incorporate phosphorus at one or more Thr, Ser, or Tyr  
20 residues, and a fatty acid, preferably palmitate, at a cysteine residue.

The present invention may employ an inhibitor of the PL scramblase activity of PL scramblase. This inhibitor may be an antisense nucleotide derived from the DNA  
25 sequence of PL scramblase. In another embodiment, the inhibitor is a peptide sequence that is a competitive inhibitor of PL scramblase activity. In another

embodiment, the inhibitor is an antibody, preferably a monoclonal antibody, raised against PL scramblase.

In another embodiment, the inhibitor works by modifying or inhibiting the post-translational  
5 modifications of the PL scramblase that are disclosed below in the Examples. For example, analysis of the primary PL scramblase sequence reveals a potential site of phosphorylation by protein kinase C or other cellular  
10 kinase (Thr<sup>161</sup>), potential sites for acylation by fatty acid, and a potential binding site for Ca<sup>2+</sup> ion provided by an EF-hand-like loop spanning residues Asp<sup>273</sup>-Asp<sup>284</sup>. These residues and motifs are conserved in the mouse PL scramblase.

In another embodiment, the inhibitor is a compound  
15 that prevents thioacylation of the protein.

In another embodiment, a mutant phospholipid scramblase is provided in which cysteine residues have been replaced by alanine or other non-conservative substitution.

20 The present invention is also a method for promoting the viability and circulating lifetime of *in vitro* stored leukocytes, lymphocytes, platelets, or red blood cells. This method comprises the steps of adding an inhibitor of PL scramblase activity to the stored blood cells.

25 The present invention is also a method for prolonging survival of transplanted cells, tissues, or organs, comprising the step of adding an inhibitor of PL scramblase activity to an organ perfusate or cell storage

medium during *in vitro* organ storage. The present invention is also a method for prolonging the survival of transplanted cells, tissues, and organs by genetically engineering the cells to be transplanted so as to reduce  
5 their expression of plasma membrane PL scramblase in order to reduce exposure of PS and other thrombogenic and pro-inflammatory phospholipids at the plasma membrane surface. In one embodiment of the present invention, the transplanted cells to be treated are hematopoietic stem  
10 cells to be used to repopulate the bone marrow by transfusion into a patient.

The present invention is also a method for diagnosing the survivability, metastatic, and invasive potential of a cancer cell or cancerous tissue or virus-  
15 infected cell by quantifying the level of cellular expression of PL scramblase and by detecting deletion or loss of function mutations in PL scramblase. For example, this quantitation may take the form of immunoblotting using an antibody to PL scramblase, an  
20 ELISA assay using an antibody to PL scramblase, flow cytometric analysis of the binding of monoclonal antibody reactive against the predicted extracellular domain of PL scramblase (residues Ser<sup>310</sup> Trp<sup>318</sup> of sequence disclosed in SEQ ID NO:2 or the equivalent residue in the conserved  
25 region of another PL scramblase) or using oligonucleotides derived from PL scramblase cDNA and the polymerase chain reaction. In one method, Northern blotting or *in situ* hybridization is performed using cDNA

of human PL scramblase as probe. In another method of the present invention, mutations in the PL scramblase gene locus are detected by DNA sequencing of the cDNA product of the polymerase chain reaction and by analysis  
5 of restriction length polymorphisms. In another method of the present invention, the quantitation is performed by isolating PL scramblase from tissue biopsy or a patient blood sample, measuring the amount of PL  
10 scramblase isolated and comparing the measurement with a control sample. The measurement may be by isolating PL scramblase from a patient tissue biopsy or blood sample and measuring via densitometry the amount of PL  
scramblase protein electrophoresed in a stained electrophoretic gel.

15

## EXAMPLES

EXAMPLE 1: Cloning of Murine PL scramblase and Identity of a Conserved Motif in Phospholipid Scramblase that is Required for Accelerated Transbilayer Movement of Membrane Phospholipids by  $\text{Ca}^{2+}$  (Zhou, et al., Biochemistry  
20 37:2356-2360, 1998).

A. Summary

Accelerated transbilayer movement of plasma membrane phospholipids (PL) upon elevation of  $\text{Ca}^{2+}$  in the cytosol plays a central role in the initiation of plasma clotting  
25 and in phagocytic clearance of injured or apoptotic cells. We recently identified a human erythrocyte membrane protein that induces rapid transbilayer movement

of PL at elevated  $\text{Ca}^{2+}$ , and presented evidence that this PL scramblase is expressed in a variety of other cells and tissues where transbilayer movement of plasma membrane PL is promoted by intracellular  $\text{Ca}^{2+}$  (Q. Zhou, et al., J. Biol. Chem. 272:18240-18244, 1997). We have now cloned murine PL scramblase for comparison to the human polypeptide (Fig. 1B): Both human and murine PL scramblase are acidic proteins ( $\text{pI}=4.9$ ) with a predicted inside-outside (type 2) transmembrane segment at the carboxyl-terminus (Fig. 5). Whereas human PL scramblase (318 AA) terminates in a short exoplasmic tail, murine PL scramblase (307 AA) terminates in the predicted membrane-inserted segment. The aligned polypeptide sequences reveal 65% overall identity, including near identity through 12 residues of an apparent  $\text{Ca}^{2+}$  binding motif (D[A/S]DNFGIQFPLD) spanning residues 273-284 (human, SEQ ID NO:2) and 271-282 (murine, SEQ ID NO:4), respectively (Fig. 5). This conserved sequence in the cytoplasmic domain of PL scramblase shows similarity to  $\text{Ca}^{2+}$ -binding loop motifs previously identified in known EF-hand structures. Recombinant murine and human PL scramblase were each expressed in *E. coli* and incorporated into proteoliposomes. Measurement of transbilayer movement of NBD-labeled PL confirmed that both proteins catalyzed  $\text{Ca}^{2+}$ -dependent PL flip/flop similar to that observed for the action of  $\text{Ca}^{2+}$  at the cytoplasmic face of plasma membranes. Mutation of residues within the putative EF hand loop of human PL scramblase resulted in loss of its

PL mobilizing function, suggesting that these residues directly participate in the  $\text{Ca}^{2+}$  induced active conformation of the polypeptide.

B. Experimental Procedures

5       Abbreviations used: PL, phospholipids(s); PC, phosphatidylcholine; PS, phosphatidylserine; NBD-PC, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-sn-glycero-3-phosphocholine; EST, expressed sequence tag; MBP, maltose binding protein;  
10      PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PCR; polymerase chain reaction.

Materials: Mouse fibroblast 5'-stretch plus cDNA library and KlenTaq polymerase were obtained from CLONTECH Laboratories. Expressed sequence Tag (EST)  
15      clone with GenBank™ accession number gb AA110551 was from American Type Culture Collection (ATCC 977052).  $\alpha$ - $^{32}\text{P}$ -dCTP was purchased from Dupont. Random Primed DNA Labeling Kit was from Boehringer Mannheim. Hybond-N Nylon membrane was from Amersham. Expression vector  
20      pMAL-C2, amylose resin and all restriction enzymes were from New England Biolabs. Wizard Kit was from Promega. Qiagen Lambda Kit was from Qiagen. Egg yolk phosphatidylcholine (PC), brain phosphatidylserine (PS) and 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-sn-glycero-3-phosphocholine (NBD-PC)  
25      were obtained from Avanti Polar Lipids. Factor Xa was from Haematologic Technologies, and Bio-Beads SM-2 were from BioRad. N-octyl- $\beta$ -D-glucopyranoside and Glu-Gly-Arg

chloromethyl ketone were from Calbiochem. Sodium dithionite (Sigma) was freshly dissolved in 1 M Tris, pH 10, at a concentration of 1 M.

Labeling of DNA Probe: The DNA insert of EST clone  
5 gb AA110551 was released by digestion with EcoRI and  
ApaI and purified by Wizard Kit. Four micrograms of  
purified DNA were labeled with 1 mCi of  $\alpha$ - $^{32}\text{P}$ -dCTP. The  
specific radioactivity of the probe was  $3.9 \times 10^8$  dpm/ $\mu\text{g}$   
DNA.

10 Isolation of Mouse PL Scramblase cDNA by Plaque

Hybridization: *E. coli* strain Y1090r was  
transformed by mouse fibroblast cDNA library ( $6 \times 10^5$  pfu)  
and poured onto 30 plates (15 cm diameter, 20,000 pfu per  
plate). Plaques were lifted onto Hybond-N Nylon  
15 membranes. After denaturation, neutralization and UV-  
cross-linking, the membranes were first prehybridized in  
a solution composed of 5X Denhardt, 5X SSC, 1% SDS, and  
200  $\mu\text{g/ml}$  herring sperm DNA for 3 hours at  $68^\circ\text{C}$ , and then  
hybridized in the same solution containing 5 ng/ml  $^{32}\text{P}$ -  
20 labeled probe for 16 hours at  $68^\circ\text{C}$ . The membranes were  
washed once with 2X SSC, 0.1% SDS, then three times with  
0.1 X SSC, 0.1% SDS for 20 minutes at  $65^\circ\text{C}$ , and exposed  
to X-ray film. Secondary plaque lifts and hybridization  
25 were carried out on 8 positive plaques at a density of  
about 100 plaques/plate. Single positive and well  
isolated plaques were picked and amplified.  $\lambda$ DNA was  
purified with Qiagen Lambda Maxi Kit.



DNA Sequencing. DNA was sequenced on an ABI DNA Sequencer Model 373 Stretch (Applied Biosystems) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer).

5        Cloning of Mouse PL Scramblase into pMAL-C2 Expression Vector. In order to express mouse PL scramblase as a fusion protein with maltose binding protein (MBP), cDNA encoding mouse PL scramblase was cloned into pMAL-C2 expression vector. PCR was performed  
10       on a mouse scramblase clone using the primers 5'TCA GAA TTC GGA TCC ATG GAG GCT CCT CGC TCA GGA AC3' (SEQ ID NO:8) with an *EcoRI* site before the ATG start codon and 5'GCT TGC CTG CAG GTC GAC CTA CAC ACA GCC TTC AAA AAA CAT G3' (SEQ ID NO:9) with a *SalI* site after the stop codon.  
15       KlenTaq polymerase was used to ensure high fidelity amplification. The PCR product was digested with *EcoRI* and *SalI*, isolated by electrophoresis, and cloned into pMAL-C2 immediately 3' of MBP. *E. coli* strain TB1 was transformed, and sequence of the cDNA insert of plasmid  
20       from a single colony was confirmed.

Expression and Purification of Mouse PL Scramblase-MBP Fusion Protein: Mouse PL scramblase was expressed as fusion protein with MBP in *E. coli* TB1 and purified on amylose resin as previously described for human PL  
25       scramblase (Q. Zhou, et al., J. Biol. Chem. 272:18240-18244, 1997). The purified fusion protein was centrifuged at 106,000 x g for 1 hour at 4°C to remove aggregated protein.

Reconstitution and Functional Activity of PL

Scramblase: Reconstitution, removal of MBP, and functional assay of PL scramblase were performed as previously described (F. Bassé, et al., J. Biol. Chem. 271:17205-17210, 1996; Q. Zhou, et al., supra, 1997; J.G. Stout, et al., J. Clin. Invest. 99:2232-2238, 1997). Routinely, 420 pmoles of protein were reconstituted with 1  $\mu$ mol of PL. To remove MBP, proteoliposomes were incubated 3 hours at room temperature with 1/40 (w/w) factor Xa. The digest was terminated by addition of 100  $\mu$ M Glu-Gly-Arg chloromethyl ketone. Proteoliposomes labeled with NBD-PC were incubated for 2 hours at 37°C in Tris buffer in the presence or absence of  $\text{CaCl}_2$ , as indicated in figure legends and diluted 25-fold in Tris buffer containing 4 mM EGTA. Initial fluorescence was recorded (SLM Aminco 8000 spectrofluorimeter; excitation at 470 nm, emission at 532 nm), 20 mM dithionite was added, and the fluorescence was continuously monitored for a total of 120 seconds. Scramblase activity was calculated according to the difference in non-quenchable fluorescence observed in presence vs absence of  $\text{CaCl}_2$ . Ionized  $[\text{Ca}^{2+}]$  was calculated using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA).

Protein Concentrations: Protein concentrations were estimated based upon optical density at 280 nm, using extinction coefficients ( $\text{M}^{-1}\text{cm}^{-1}$ ) of 39,000 (PL

scramblase), 64,500 (MBP), and 105,000 (PL scramblase-MBP fusion protein).

Mutagenesis of PL Scramblase: Human PL scramblase amino acid residues in EF-hand  $\text{Ca}^{2+}$ -binding motif at positions of Asp<sup>273</sup>, Asp<sup>275</sup>, Phe<sup>277</sup>, Ile<sup>279</sup>, Phe<sup>281</sup> and Asp<sup>284</sup> were mutated to Ala with oligonucleotide-directed mutagenesis by two rounds of PCR. PL scramblase-pMAL-C2 was selected as template, and the first round of PCR was performed with pairs of a complementary oligonucleotide primer containing the point mutation plus a primer complementary to a site near the ATG initial codon or TAG stop codon. PCR products were purified by Wizard kit. Full-length mutated PL scramblase cDNA was obtained by overlapping PCR and cloned back into pMAL-C2 vector. After confirmation of correct DNA sequence the mutants were recombinantly expressed in *E. coli* as described above and analyzed by SDS-PAGE.

### C. Results and Discussion

Isolation of cDNA of Mouse PL Scramblase. Murine EST clones in GenBank containing putative PL scramblase sequence were identified by a Blast homology search using the human PL scramblase cDNA. Among several clones exhibiting significant homology, a 403 bp Stratagene mouse kidney clone (gb accession number AA110551) with 79% nucleotide sequence identity to human PL scramblase was selected and this clone was used to probe a mouse fibroblast cDNA library. Eight positive clones were identified after two rounds of plaque hybridization. Two

of the eight clones were sequenced yielding 1354 bp and 1529 bp, respectively. Alignment revealed 1261 bp of overlapping sequence that spanned an open reading frame of 921 bp and specified a total of 1622 bp of unique cDNA sequence (SEQ ID NO:3).

SEQ ID NO:4 represents the open reading frame of the translated sequence of SEQ ID NO:3 (see Fig. 1B). The deduced mouse PL scramblase cDNA encodes a 307 residue protein with a molecular weight of 33.9 kDa and a theoretical pI = 4.9, similar to values obtained for the human protein (318 residues, 35.1 kDa; pI=4.9; ref. (Q. Zhou, et al., supra, 1997)). The overall identity of the mouse and human PL scramblase is 64.8%, with the most divergent sequence generally contained in the N-terminal portion of the polypeptide (Fig. 5). Fig. 5 depicts the alignment of protein sequences of mouse and human PL scramblase. Alignment between mouse (MUR) and human (HUM) PL scramblase was performed by FASTA program using the Smith-Waterman algorithm. (W.R. Pearson and D.J. Lipman, Proc. Natl. Acad. Sci. USA 85:2444-2448, 1988) Sequence of human PL scramblase is contained in GenBank™ accession number AF008445. Amino acid identities ( : ) or similarities ( . ) between the two sequences are indicated. Also indicated are the residues comprising a predicted inside-out transmembrane domain (MUR 289-307, HUM 291-309; double underline), and the 12 residues of the acidic loop of a putative EF-hand (MUR 271-282, HUM 273-284; single underline).

In both proteins, a single 19 residue transmembrane helix is predicted at the carboxyl terminus, exhibiting a strongly preferred inside-to-outside orientation. Whereas the mouse protein terminates immediately after this conserved transmembrane helix, the human PL scramblase contains an additional nine residues, implying that the short exoplasmic peptide in human PL scramblase is non-essential to function. Homology motifs conserved in both proteins include a potential site for protein kinase C phosphorylation (Thr<sup>159</sup> in mouse, Thr<sup>161</sup> in human) and a potential Ca<sup>2+</sup>-binding EF-hand loop motif adjacent to the transmembrane helix (residue Asp<sup>271</sup> to Asp<sup>282</sup> in mouse and residues Asp<sup>273</sup> to Asp<sup>284</sup> in human). The cytoplasmic orientation of this protein and the proximity of this putative Ca<sup>2+</sup>-binding domain to the segment of polypeptide that is inserted into the plasma membrane are consistent with the proposed activity of this protein *in situ*, where Ca<sup>2+</sup> acting directly at the endofacial membrane surface is known to initiate the rapid transbilayer movement of plasma membrane PL (P. Williamson, *et al.*, Biochemistry 31:6355-6360, 1992; R.F.A. Zwaal and A.J. Schroit, Blood 89:1121-1132, 1997; F. Bassé, *et al.*, J. Biol. Chem. 271:17205-17210, 1996; D.L. Bratton, J. Biol. Chem. 269:22517-22523, 1994; B. Verhoven, *et al.*, Biochim. Biophys. Acta 1104:1523, 1992).

Functional Activity of Recombinant Mouse PL Scramblase. In order to confirm that the cDNA identified

as mouse PL scramblase encodes a protein of similar function to that identified in human, the human and mouse proteins were each expressed in *E. coli*, purified, and reconstituted in proteoliposomes for measurement of PL mobilizing activity. Mouse or human PL scramblase-MBP fusion protein (420 pmoles) was reconstituted into PC/PS liposomes (1  $\mu$ mol total PL), respectively, MBP was removed by digestion of the proteoliposomes with factor Xa, and PL scramblase activity was determined as described under "Experimental Procedures" and plotted as a function of external free  $[Ca^{2+}]$ . The results of this experiment indicate that recombinant mouse PL scramblase mediated a  $Ca^{2+}$ -dependent transbilayer movement of membrane PL with a specific activity and affinity for  $Ca^{2+}$  indistinguishable from that observed for the recombinant human protein.

Mutational Analysis of a Putative Conserved EF-Hand Motif. As noted above, the deduced protein sequence of mouse and human PL scramblase reveals an extensive segment of highly conserved sequence extending through residue Glu<sup>306</sup> (in human; corresponding to Glu<sup>304</sup> in mouse; Fig. 5). The predicted secondary structure through this portion of the protein reveals that it contains two short alpha-helical segments near the C-terminus that are separated by a 12-residue acidic loop. In both proteins (human and mouse), the C-terminal alpha helix represents a predicted transmembrane segment with a strongly-preferred inside-to-outside orientation, whereas sequence

contained within the adjacent 12-residue acidic loop conforms in-part to a consensus sequence that is characteristic of an EF-hand  $\text{Ca}^{2+}$ -binding loop motif (S. Nakayama and R.H. Kretsinger, Annu. Rev. Biophys. Biomol. Struct. 23:473-507, 1994). In this motif, residues in positions 1, 3, 5, 7, 9 and 12 of the loop contribute to octahedral coordination of the  $\text{Ca}^{2+}$  ion, with the residues in position 1[Asp], 3[Asp, Asn, or Ser] and 12 [Asp or Glu] being those most highly conserved.

10 In order to gain insight into whether this segment of the protein might be directly involved in the  $\text{Ca}^{2+}$ -dependent reorganization of membrane PL mediated by PL scramblase, we expressed mutant human PL scramblase with Ala substitutions at positions corresponding to residues  
15 1 (Asp<sup>273</sup>), 3 (Asp<sup>275</sup>), 5 (Phe<sup>277</sup>), 7 (Ile<sup>279</sup>), 9 (Phe<sup>281</sup>), and 12 (Asp<sup>284</sup>) of this putative 12 residue EF-hand loop.

Fig. 6 illustrates PL scramblase activity as a function of mutational analysis of putative EF hand loop motif contained in human PL scramblase. Wild-type (WT)  
20 and mutant constructs of human PL scramblase were expressed as fusion proteins with MBP in *E. coli*, purified, and reconstituted in proteoliposomes. After release of MBP by incubation with factor Xa, PL scramblase activity was assessed (see "Experimental  
25 Procedures"). For each mutant construct, the residues in human PL scramblase that were replaced by Ala are indicated on the *abscissa*. PL scramblase activity (*ordinate*) was measured in presence of 2 mM  $\text{CaCl}_2$ , and in

each case was normalized to the activity of WT human PL scramblase ( $11.76 \pm 0.44\%$  of total NBD-PC flipped), with correction for the non-specific transbilayer movement of NBD-PC ( $0.20 \pm 0.08\%$  of total NBD-PC flipped) measured in PL vesicles lacking added protein. Error bars indicate mean  $\pm$  SD of three independent measurements performed with each mutant construct. Fig. 6 illustrates the data of single experiment, representative of two separate experiments so performed.

As illustrated by Fig. 6, Ala substitution at any of these positions reduced PL scramblase function, with mutation at Asp<sup>275</sup> resulting in complete inactivation of the Ca<sup>2+</sup>-dependent response. In those mutant polypeptides showing partial retention of activity, reduced response to Ca<sup>2+</sup> was related in-part to an apparent reduction in avidity for Ca<sup>2+</sup> (Fig. 7).

Fig. 7 illustrates the Ca<sup>2+</sup>-dependence of mutant human PL scramblase. PL scramblase activity of wild-type (WT) and selected mutant constructs of Fig. 6 was determined as described in "Experimental Procedures" and plotted as a function of external free [Ca<sup>2+</sup>]: WT(●); Asp<sup>273</sup>(□); Phe<sup>277</sup>(Δ); Ile<sup>279</sup>(◇); Phe<sup>281</sup>(○); Asp<sup>284</sup>(∇). The data are corrected for non-specific transbilayer migration of NBD-PC in the absence of free [Ca<sup>2+</sup>]. Data of single experiment. The results described in Fig. 7 suggest that residues contained in the putative EF-hand loop spanning Asp<sup>273</sup>-Asp<sup>284</sup> are critical to the function of PL scramblase, presumably for coordination of Ca<sup>2+</sup> as



required to induce the PL transporting state of the protein. It remains to be determined what conformational changes are induced in the polypeptide in the presence of  $\text{Ca}^{2+}$ , including potential reorientation of helical segments flanking the putative  $\text{Ca}^{2+}$  binding loop, that might contribute to the accelerated transbilayer movement of membrane phospholipids.

EXAMPLE 2: Inactivation of Human PL Scramblase by Treatment With the Thiolester Cleaving Reagent, Hydroxylamine (Zhao, et al., Biochemistry 37:6361-6366, 1998).

A. Summary

Incubation of human erythrocyte PL scramblase with hydroxylamine under conditions known to favor hydrolysis of protein cysteinyl-fatty acyl bonds was found to cause near complete loss of PL scramblase's function in promoting movement of PL between membrane leaflets. These data suggest that for normal activity, the PL scramblase polypeptide requires post translational modification through addition of a thiolester-linked fatty acid. Furthermore, these data imply that methods that either prevent cellular acylation of the polypeptide, or that cleave cysteinyl thiolester linkages, will effectively inhibit endogenous PL scramblase activity.

B. Methods

Protein purification. PL scramblase was purified from human erythrocyte ghost membranes as previously

described (F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997).

Treatment with Hydroxylamine. PL scramblase was incubated 1 hour at room temperature in 1 M hydroxylamine, 25 mM octylglucoside, 1 M Tris-HCl at pH 7.4. Match control samples of the protein were identically incubated under these conditions, omitting hydroxylamine. After incubation, samples were dialyzed and reconstituted into PL proteoliposomes for assay of PL scramblase activity.

Membrane reconstitution and assay. PL scramblase was reconstituted into proteliposomes and activity determined as previously described (F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997).

#### 15 C. Results and Discussion

As shown in Fig. 11, incubation with hydroxylamine resulted in nearly complete inactivation of PL scramblase. Fig. 11 illustrates inactivation of PL scramblase function by treatment with hydroxylamine. The figure shows loss of functional activity of human erythrocyte PL scramblase due to treatment with the thioester cleaving reagent hydroxylamine. Identical aliquots of human erythrocyte PL scramblase in 50 mM octylglucoside were incubated 1 hour room temperature in either 1 M hydroxylamine, pH 7.2 (solid bar) or 1 M Tris-HCl, pH 7.2 (open bar). After treatment, each sample was reconstituted into proteoliposomes and PL scramblase activity measured using NBD-phosphatidylcholine as probe

according to methods previously described (Basse, et al.,  
supra, 1996; Stout, et al., supra, 1997; Zhou, et al.,  
supra, 1997). Error bars denote mean  $\pm$  SD of seven  
independent experiments. Significance by paired  
5 students' t-test,  $p < 10^{-6}$ . (See Methods, Example 3.)

Because the conditions of incubation (neutral pH)  
were chosen to favor specific cleavage of cysteinyl  
thioester bonds without disulfide bond reduction, these  
results imply an essential thioester linkage within the  
10 protein. In a membrane-associated protein with  
cytoplasmic Cys residues, such as found in erythrocyte PL  
scramblase, this thiolester bond is normally provided by  
palmitic acid in ester linkage to one or more cysteinyl  
thiols. (H. Schroeder, et al., J. Cell Biol. 134:647-660,  
15 1996; M. Stauffenbiel, J. Biol. Chem. 263:13615-13622,  
1988; C.A. Wilcox, et al., Biochemistry 26:1029-1036,  
1987). Whereas the possibility of disulfide reduction by  
hydroxylamine cannot be excluded, it is important to note  
that (1) virtually all cysteine residues in PL scramblase  
20 are normally exposed to cytoplasmic reducing agents such  
as glutathione, and disulfide bond formation is therefore  
not anticipated and (2) The absence of any functionally-  
important disulfide bonds in PL scramblase can be assumed  
based on the retention of normal PL scramblase activity  
25 when the protein was incubated in various reducing  
agents, including dithiothreitol (F. Bassé, et al.,  
supra, 1996; J.G. Stout, et al., supra, 1997). Thus  
these data suggest that PL scramblase polypeptide

requires post-translational modification through addition of a thiolester-linked fatty acid for its normal function in the plasma membrane. Furthermore, these data imply that reagents that either prevent cellular acylation of the polypeptide, or, reagents that cleave cysteinyl thiolester linkages, will effectively inhibit endogenous PL scramblase activity.

The presence of covalently-linked palmitic acid in PL scramblase was directly confirmed by metabolic labeling of the protein with [ $^3\text{H}$ ]-palmitate. Human Raji cells, which we have shown to contain low amounts of endogenous PL scramblase, were transformed with plasmid encoding the human PL scramblase as a fusion protein with green fluorescent protein (GFP), and metabolically labeled with [ $^3\text{H}$ ]-palmitate. Immunoprecipitation with antibody to GFP revealed the covalent incorporation of [ $^3\text{H}$ ] in GFP-PL scramblase fusion protein, whereas no radioactivity was associated with GFP (Fig. 12).

Fig. 12 illustrates that metabolic labeling of PL scramblase with [ $^3\text{H}$ ]-palmitate reveals covalent thioester-linked fatty acid. The presence of cysteinyl-linked palmitate in PL scramblase was confirmed by metabolic labeling of pEGFP-PL scramblase-transfected Raji cells (GFP-PLS) or identically-treated vector-only controls (GFP) with exogenous [ $^3\text{H}$ ]-palmitate. Cells were incubated ( $10^7$  cell/ml, 1 hour,  $37^\circ\text{C}$ ) with  $200\ \mu\text{Ci/ml}$  [ $^3\text{H}$ ]-palmitate (Dupont NEN, spec. act.  $60\ \text{Ci/mmol}$ ) in RPMI-1640 containing 20% dialyzed fetal bovine serum and 5 mM

sodium pyruvate. Cells were then harvested, washed and membrane proteins ( $10^8$  cells/ml) extracted into 1% triton X100, 5 mM EDTA, 20 mM leupeptin, 20 mM M-ethylmaleimide, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride in tris-buffered saline, pH 7.4. Insoluble material was removed by centrifugation and the supernatants precleared by incubation with 10  $\mu$ g/ml normal rabbit IgG and 30  $\mu$ l protein A sepharose (Pharmacia). The GFP and GFP-PLS proteins were then immunoprecipitated overnight with 50  $\mu$ g/ml affinity purified rabbit anti-GFP (Clontech) and staph A sepharose. The beads were exhaustively washed with 1% triton X100 and GFP vector control (Lanes 1 and 2) and the GFP-PL scramblase fusion protein (Lanes 3 and 4) each eluted from staph A sepharose into 5% SDS-PAGE sample buffer. Each sample was divided and incubated 1 hour with either 1 M hydroxylamine, pH 7.2 (+) or 1 M Tris, pH 7.2 (-) and then subjected to polyacrylamide gel electrophoresis under non-reducing conditions (4-20% polyacrylamide; NOVEX). UPPER PANEL: Western blotting was performed with murine monoclonal antibody against human PL scramblase (anti-PLS). MIDDLE PANEL: Western blotting performed with murine monoclonal antibody against green fluorescent protein (anti-GFP; Clontech)). LOWER PANEL shows fluorogram of radioactivity from  $^3\text{H}$ . Fluorography of the SDS-PAGE gel was performed after fixation in 50% ethanol, 5% acetic acid, and the washed gel soaked in Enlightning scintillant (Dupont-NEN) before drying and exposure to preflashed Kodak X-AR5 film (-

80°C, 4 days). Note radioactivity in GFP-PL scramblase (see autoradiography of ~65 kDa protein band, lane 4) that is released from GFP-PL scramblase by treatment with hydroxylamine (see radioactivity at dye front, lane 3).

- 5 Data of single experiment, representative of two independent experiments.

The identity of the covalently-bound [<sup>3</sup>H] in PL scramblase as a thioester-linked [<sup>3</sup>H]-palmitate was confirmed by hydroxylamine cleavage and by thin layer  
10 chromatography (TLC) of the liberated fatty acid (Fig. 13).

Fig.13 illustrates that TLC analysis of [<sup>3</sup>H]-fatty acid in PL scramblase. The GFP-PLS protein band at ~65 kDa of the previous figure was sliced from the wet gel  
15 and the gel soaked in 30% methanol, 10% acetic acid, washed exhaustively with 50% methanol, and then lyophilized. To liberate covalently-bound fatty acids, the dried gel was incubated overnight in 1.5N NaOH. Following acidification with HCl (pH<2), the released  
20 fatty acids (1 ml) were extracted by addition of 3.75 ml chloroform:methanol (1:2 v:v). To separate the fatty acids into a chloroform phase, 1.25 ml chloroform and 1.25 ml H<sub>2</sub>O were then added, and the bottom layer recovered and dried under nitrogen. The dried pellet was  
25 taken up in chloroform:methanol (2:1, v:v) and spotted onto KC18 silica gel (Whatman 4801-425). The chromatograph was developed in acetonitrile:acetic acid (9:1, v:v) and air dried. The dried plate was sprayed

with Enhance (Dupont-NEN) and radioactivity was detected by fluorography on preflashed Kodak XAR-5 film (-80°C, 4 days). Radioactivity liberated from GFP-PLS (lane 2) is compared to matched gel slice from GFP vector control (lane 3), using [<sup>3</sup>H]-palmitate as standard (standards applied in lanes 1 and 4). All samples were spotted at position OR; SF indicates position of the solvent front. Data of single experiment.

Thus, PL scramblase is palmitoylated at one or more cysteine residues. We also have evidence that such acylation is required for normal function of PL scramblase protein. As noted above, removal of palmitate through hydrolysis of thioester bonds with hydroxylamine was found to abolish PL scramblase's phospholipid mobilizing function (Fig. 11).

EXAMPLE 3: Plasma Membrane Expression of Phospholipid Scramblase Regulates Ca<sup>2+</sup> Induced Movement of Phosphatidylserine to the Cell Surface: Alteration of Phosphatidylserine Exposure In Human Lymphoblasts Through Stable Transfection with PL Scramblase cDNA.

A. Summary

In order to determine whether PL scramblase is responsible for the rapid movement of PS from inner-to-outer plasma membrane leaflets in other cells exposed to elevated cytosolic [Ca<sup>2+</sup>]<sub>c</sub>, we analyzed how induced movement of PS to the surface related to cellular content of PL scramblase. Exposure to Ca<sup>2+</sup> ionophore A23187 resulted in rapid PS exposure in those cells high in PL

scramblase (K-562, HEL, 293T, and EBV-transformed lymphocytes), whereas this response was markedly attenuated in cells with low amounts of the protein (Raji, MOLT-4, HL-60). To confirm this apparent correlation between PL scramblase expression and PS egress at elevated  $[Ca^{2+}]_c$ , Raji cells were transfected with PL scramblase cDNA in pEGFP-C2, and stable transformants expressing various amounts of rGFP-PL scramblase fusion protein obtained. Clones expressing rGFP-PL scramblase showed plasma membrane-localized fluorescence and elevated PL scramblase antigen whereas clones expressing rGFP alone (transfected with pEGFP-C2 without insert) showed only cytoplasmic fluorescence and served as controls. In absence of ionophore, expression of rGFP-PL scramblase had no effect on cell viability or background PS exposure. In response to A23187, clones expressing GFP-PL scramblase exhibited markedly accelerated movement of PS to the cell surface when compared to A23187-treated clones expressing GFP with PS movement to the cell surface increasing with amount of rGFP-PL scramblase expressed. These data indicate that transfection with PL scramblase cDNA promotes  $[Ca^{2+}]_c$ -dependent movement of PS to the cell surface and suggest that this protein normally mediates redistribution of plasma membrane phospholipids in activated, injured, or apoptotic cells exposed to elevated  $[Ca^{2+}]_c$ .



B. Materials and Methods

Materials. All restriction enzymes were from New England BioLabs, Inc. (Beverly, MA). KlenTaq polymerase and pEGFP-C2 vector were from CLONTECH Laboratories (Palo Alto, CA). Bovine coagulation factor Va (FVa), factor Xa (FXa), prothrombin and dansylarginine-N-(3-ethyl-1,5-pentanediy1)amide were from Haematologic Technologies, Inc. (Essex Junction, VT). Chromogenic thrombin substrate S2238 was from DiaPharma Group, Inc. (Franklin, OH). Human  $\alpha$ -thrombin was a generous gift from Dr. John W. Fenton (Albany, NY). OPTI-MEM and geneticin were from Life Technologies (Gaithersburg, MD). Fetal bovine serum, RPMI 1640, Cell Dissociation Solution, Hank's Balanced Salt Solution (HBSS), Protein A Sepharose-CL4B, leupeptin, and BSA were from Sigma Chemical Co. (St. Louis, MO). UltraLink Iodoacetyl resin and SuperSignal ULTRA Chemiluminescence Kit were from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

Cell culture: Human cancer cell lines erythroleukemic HEL, promyelocytic leukemia HL-60, chronic myelogenous leukemia K562, lymphoblastic leukemia MOLT-4, acute T-cell leukemia Jurkat, Burkitt's lymphoma Raji, and megakaryocytic DAMI were from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 containing 10% fetal bovine serum. EBV-transformed cell line W9 established from peripheral B-lymphocytes of

a normal donor was maintained as previously described (H. Kojima, et al., 1994).

Antibodies: Anti-GFP: murine monoclonal antibody against green fluorescent protein (GFP) was from CLONTECH Laboratories. Anti-FVa: murine monoclonal antibody V237 reactive against human or bovine factor Va light chain was the generous gift of Dr. Charles T. Esmon (Oklahoma Medical Research Fndn, Oklahoma City, OK). Anti-PL Scramblase-E306-W318: Rabbit antibody raised against the carboxyl terminal peptide sequence E306-W318 of human PL  
10 scramblase has previously been described (Q. Zhou, et al., supra, 1997). The IgG fraction was isolated on protein A-Sepharose-CL4B and the peptide-reactive antibody purified by affinity chromatography on peptide  
15 [Cys]-ESTGSQEQKSGVW (SEQ ID NO:5) coupled to UltraLink Iodoacetyl resin.

Plasmid Construction: Human PL scramblase cDNA insert was released from plasmid pMAL-C2-PL scramblase (Q. Zhou, et al., supra, 1997) by double cutting with  
20 *EcoRI* and *SalI*, respectively, and then ligated into pEGFP-C2 vector using the same restriction site. The pEGFP-C2-PL scramblase plasmid was amplified from single clones in *E. coli* strain Top 10, and the orientation and reading frame of the insert confirmed by sequencing on an  
25 ABI DNA Sequencer Model 373 Stretch (Perkin Elmer-Applied Biosystems, Foster City, CA) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit.

Transfection of Raji cells with pEGFP-PL scramblase.

1.6 x 10<sup>7</sup> Raji cells were electroporated with 160 µg plasmid DNA (pEGFP-C2-PL scramblase or pEGFP-C2) in a total volume of 0.8 ml OPTI-MEM, using Gene Pulse  
5 Electroporator (Bio-Rad Laboratories, Hercules, CA) set at 450 V, 500°F. After 48 hours in culture, 1.5 mg/ml geneticin was added to the medium and continuously maintained for 4 weeks. Stable transformants exhibiting GFP fluorescence were sorted by flow cytometry (FACStar,  
10 Becton-Dickinson Immunocytometry Systems, San José, CA) using an FL1 sorting gate. The FL1-positive cells were dilutionally cloned in 96 well culture plates. pEGFP-PL scramblase transformants expressing the 62 kDa GFP-PL  
scramblase fusion protein were identified by Western  
15 blotting with anti-GFP and with anti-PL-306-W318 antibodies. Western blotting of pEGFP-C2 transformants (without insert) confirmed presence of 27 kDa GFP. Clones expressing various amounts of GFP-PL scramblase were each expanded for functional assay, along with  
20 comparable GFP-expressing clones serving as controls.

Fluorescence Microscopy. Cell clones transfected with pEGFP-C2-PL scramblase or pEGFP-C2 were deposited on glass microscopy slides using a Cytospin 3 (Shandon,  
Inc., Pittsburgh, PA). Phase contrast and fluorescence  
25 microscopy was performed with a ZEISS AXIOSKOP microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence, and images were recorded with a MC100 camera system. The exposure times for photography of

fluorescence was 80-200 seconds under automatic control using Kodak Ektachrome 1600 film.

Western Blot Analysis. Western blotting of GFP and PL scramblase antigens was performed using  $1.5 \times 10^6$  cells per lane. After washing in HBSS, supernatants were removed, and the cell pellets extracted with 2% (v/v) NP-40 in 5 mM EDTA, 50 mM benzamidine, 50 mM N-ethyl maleimide, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin in HBSS. After removal of insoluble material (250,000 x g, 30 minutes, 4°C), the samples were denatured at 100°C in 10% (w/v) SDS sample buffer containing 2%  $\beta$ -mercaptoethanol. Following SDS-PAGE and transfer to nitrocellulose, the blocked membrane was incubated with either 10  $\mu$ g/ml of rabbit anti-PL scramblase-E306-W318, or 1/10,000 dilution of mouse anti-GFP. The blots were developed with the horseradish peroxidase conjugate of either goat anti-rabbit IgG or goat anti-mouse IgG, respectively, using SuperSignal ULTRA chemiluminescence.

Measurement of cell surface PS. Calcium ionophore-induced exposure of PS on the surface of all cell lines analyzed was detected by the specific binding of coagulation factor Va (light chain) as previously described (P.J. Sims, et al., J. Biol. Chem. 263:18205-18212, 1988; H. Kojima, et al., J. Clin. Invest. 94:2237-2243, 1994). Briefly, cells were washed twice to remove serum proteins and suspended ( $2 \times 10^6$  cells/ml) at 37°C in RPMI 1640 supplemented with 0.1% BSA, 20 mM HEPES, and

adjusted to 1.2 mM free  $[Ca^{2+}]$ . At time=0, A23187 (0 or 2  $\mu$ M final concentration) was added from 1 mM stock solution in DMSO, and at times indicated in figure legends, the reaction was stopped by addition of 6 mM EGTA. PS exposed on the cell surface at each time point was detected by incubating (10 minutes, room temperature) 50  $\mu$ l of the cell suspension with 10  $\mu$ g/ml FVa, followed by 10  $\mu$ g/ml anti-FVa, to detect the cell-bound FVa light chain. After staining with 10  $\mu$ g/ml Tri-Color conjugated goat anti-mouse IgG (CALTAG Laboratories, Burlingame, CA), single-cell fluorescence was quantitated by flow cytometry (FL3 channel, FACScan, Becton Dickinson Immunocytometry Systems). Use of Tri-Color conjugate to detect cell-bound FVa enabled simultaneous measurement of cell-associated GFP fluorescence in cell lines transformed with the pEGFP-C2 expression plasmid (fluorescence of GFP detected in FL1 channel). In experiments in which cell lysis was monitored by uptake of propidium iodide, cells were stained for bound FVa with FITC-conjugate of goat anti-mouse IgG (FL1 channel) substituting for Tri-Color conjugate, and propidium iodide was detected in FL3 channel. Propidium iodide (0.5  $\mu$ g/ml) was added immediately before dilution for flow cytometry.

Prothrombinase Assay. Prothrombinase activity of Raji cells was determined by modification of methods previously described for platelets, using the chromogenic thrombin substrate S2238 (P.J. Sims, et al., supra,

1988). Briefly,  $1 \times 10^5$  Raji cells (transfected with either pEGFP-C2 or pEGFP-C2-PL scramblase) were suspended in 200  $\mu$ l HBSS containing 1% BSA in the presence of 2 nM FVa, 1.4  $\mu$ M prothrombin 2.5 mM  $\text{CaCl}_2$ , and 4  $\mu$ M dansylarginine-N-(3-ethyl-1,5-pentanediy1)amide (to inhibit feed-back activation by thrombin), and incubated at 37°C.  $\text{Ca}^{2+}$ -ionophore A23187 (2  $\mu$ M), or DMSO (as solvent control) was added, and prothrombin conversion was initiated by addition of 2 nM Fxa. Thrombin generation was stopped after 2 minutes by dilution into 10 mM EGTA and samples were stored on ice. Aliquots were transferred to a 96-well plate, and thrombin generated was assayed in TBS containing 1% BSA in presence of 150  $\mu$ M S2238 by monitoring time-dependent changes in absorbance at 405 nm using a Thermo<sub>max</sub> plate reader (Molecular Devices, Sunnyvale, CA). Thrombin activity was calculated using purified thrombin as standard.

### C. Results

#### Analysis of PL scramblase in various human cell

20 lines. Proteoliposomes reconstituted with erythrocyte PL scramblase exhibit accelerated transbilayer movement of fluorescent phospholipids in response to added  $\text{Ca}^{2+}$ , similar to the observed effect of calcium on the endofacial surface of the red cell membrane (Q. Zhou, et al., supra, 1997; J.G. Stout, et al., J. Clin. Invest. 29:2232-2238, 1997; Bassé, et al., J. Biol. Chem. 271:17205-17210, 1996). In order to determine whether this same protein is responsible for mediating the

accelerated egress of plasma membrane PS that is observed under conditions of elevated cytosolic  $[Ca^{2+}]_c$ , we undertook to determine whether the level of expression of PL scramblase in various human cell lines correlated to the induced movement of PS to the surface of these cells. When challenged with a calcium ionophore, human cell lines exhibit considerable differences in the extent to which PS is mobilized to the cell surface. Among the cells tested, Raji, HL60, and Dami were notably unresponsive to A23187, whereas HEL, W9 (an EBV-transformed normal B-lymphocyte), and Jurkat showed notably robust responses. This apparent cell type-specific variability in response to induced elevation of  $[Ca^{2+}]_c$  was consistently maintained through many months of passage in culture, suggesting it reflected an inherent property of each cell line. As shown in Fig. 8, we also observed considerable differences in the content of PL scramblase protein among these various cell lines, and the sensitivity of these various cell lines to induced exposure of plasma membrane PS (lower panel) generally correlated with the amount of cellular PL scramblase protein detected by Western blotting (upper panel): Those cell lines that were most responsive to induced elevation of  $[Ca^{2+}]_c$  (HEL, W9, Jurkat) also expressed greatest amounts of PL scramblase antigen, whereas cell lines with attenuated response to  $[Ca^{2+}]_c$  (Raji, HL60, Dami) contained relatively little of this protein. Cell lines Molt-4 and K562 showed intermediate responses to

elevated  $[Ca^{2+}]_i$  and expressed intermediate levels of PL scramblase antigen.

Fig. 8 depicts western blot analysis of PL scramblase in various human cell lines. Constitutive expression of PL scramblase was analyzed in the human cell lines indicated. Upper Panel: Results obtained by Western blotting with antibody specific for PL scramblase carboxyl terminal residues E306-W318 (see *Materials & Methods*). Each lane contains the total protein extract of  $1.5 \times 10^6$  cells. Lower Panel: Cumulative results of three separate experiments performed as follows: The cells indicated were washed and suspended at 37°C in the presence of 1.2 mM free  $Ca^{2+}$ , and 2  $\mu$ M A23187 was added. At times shown (*abscissa*), EGTA was added and cells analyzed for surface exposed PS as detected by cell-bound FVa light chain (see *Materials and Methods*). Data plotted represent the mean increase ( $\pm$  SD) in number of cells that stained positive for surface PS after 5 minutes incubation with ionophore, after correction for initial background of PS-positive cells before addition of ionophore (time=0). Background number of cells that exposed PS in absence of ionophore was always <15% except in case of HEL, where this background ranged between 15-30%.

These relatively large differences in cell line-specific expression of this protein was also consistently observed despite repeated passage in culture, and was found to correspond to marked differences in level of



specific mRNA as detected by Northern blotting with PL  
scramblase cDNA (Q. Zhou, et al., supra, 1997), and data  
not shown). We also noted that those cell lines with the  
highest content of PL scramblase generally exhibited a  
5 higher background of PS exposed on the surface in absence  
of added ionophore. This was most notable for HEL for  
which approximately 15-30% of the cells were consistently  
found to expose PS prior to addition of A23187 (see  
Discussion).

10 Membrane changes underlying ionophore response. In  
order to determine whether the increase in PS exposure in  
ionophore-treated cells reflected facilitated movement of  
PS from inner to outer leaflets of the plasma membrane,  
or, greater sensitivity of the plasma membrane to lytic  
15 disruption, FVa binding to the cell surface was monitored  
simultaneously with uptake of propidium iodide, as a  
measure of cell lysis. As illustrated for the human B-  
lymphocyte lines W9 (high content of PL scramblase) and  
Raji (low content of PL scramblase), the induced movement  
20 of PS to the cell surface was found to precede uptake of  
propidium iodide, suggesting that the elevation of  $[Ca^{2+}]_i$   
induces a collapse of transmembrane PL asymmetry before  
onset of lysis. In the case of Raji cells which are  
virtually devoid of PL scramblase (see Fig. 8), a general  
25 insensitivity of the plasma membrane to either ionophore-  
induced PS exposure or to lysis was also apparent.

Transfection of the Raji cell line with pEGFP-C2-PL  
scramblase. In order to confirm that the extent to which

PS moves to the cell surface with elevation of  $[Ca^{2+}]_c$ , actually depends upon the plasma membrane content of PL scramblase, we stably transformed Raji, a cell line exhibiting low endogenous PL scramblase expression by

5 transfection with plasmid pEGFP-C2-PL scramblase. This plasmid expresses PL scramblase as a fusion protein with green fluorescent protein (GFP), facilitating flow cytometric sorting of transformants for subsequent cloning and detection of the expressed recombinant

10 protein in selected clones. The decision to attach GFP to the amino terminus of PL scramblase was based on prior evidence that the carboxyl terminus of the protein is membrane inserted and essential for function, and the observation that other amino terminal fusion constructs

15 of PL scramblase expressed in *E. coli* retained the same activity of the unmodified PL scramblase polypeptide when reconstituted in proteoliposomes (Q. Zhou, et al., supra, 1997), and unpublished data). The expression of the full-length GFP-PL scramblase fusion protein in selected

20 transformed clones was confirmed by Western blotting with antibody specific for GFP, and with antibody raised against peptide sequence of the carboxyl terminus of human PL scramblase. As illustrated by fluorescence micrographs shown in Fig. 9, clones that expressed the

25 GFP-PL scramblase fusion protein showed a distinct rim appearing pattern of fluorescence, consistent with trafficking of GFP-PL scramblase to the plasma membrane. Fig. 9 illustrates fluorescence micrographs of GFP-PL

scramblase transformed Raji cells. Fluorescence photomicrography of GFP fluorescence expressed in the transformed Raji clones was performed as described in *Materials and Methods*. Fig 9A shows fluorescence of cells expressing GFP; Fig. 9B shows cells transfected with pEGFP-C2-PL scramblase plasmid and expressing GFP-PL scramblase fusion protein. Data of single experiment, representative of results obtained for all clones transfected with either pEGFP-C2 or pEGFP-C2-PL scramblase. By contrast, clones that expressed GFP alone exhibited diffuse fluorescence throughout the cytoplasm, with no obvious staining of the plasma membrane. These data provide the first direct evidence that PL scramblase cDNA encodes a protein that predominantly trafficks to the plasma membrane under normal conditions of cell growth.

Analysis of PS mobilizing function in GFP-PL scramblase transformants. After geneticin selection, clonal populations of transformed Raji cells expressing comparable levels of either GFP-PL scramblase or GFP (transformed with pEGFP-C2 lacking insert) were analyzed for their capacity to mobilize PS to the cell surface. In response to an A23187-induced elevation of  $[Ca^{2+}]_c$ , transformants expressing the GFP-PL scramblase fusion construct showed a marked increase in both the rate and extent that PS became exposed on the cell surface, when compared to either the identically-treated parental Raji cell line or to GFP-expressing clones transformed with

pEGFP-C2 vector alone. As was also evident from these data, in the absence of ionophore, we consistently noted a small but reproducible increase in the background level of PS exposure in transformants expressing GFP-PL  
5   scramblase protein, when compared to either the parental Raji cell lines or to GFP-expressing clones transformed with vector alone.

Induction of membrane procoagulant function through expression of PL scramblase. In order to confirm that  
10   the increased expression of FVa binding sites detected upon activation of GFP-PL scramblase transformed clones reflected an increase in the procoagulant (clot-promoting) properties of the plasma membrane of these cells, the capacity of GFP-PL scramblase transformed  
15   cells to provide catalytic membrane surface for the prothrombinase (FVaXa) enzyme complex was compared to clones expressing GFP alone. These data confirmed that expression of recombinant PL scramblase in the Raji cell line was also accompanied by an increase in cell capacity  
20   to catalyze the prothrombinase reaction upon entry of calcium into the cytosol.

Level of expression of PL scramblase regulates capacity of to mobilize PS to the cell surface. In order to confirm the apparent correlation between endogenous  
25   cell content of PL scramblase and plasma membrane sensitivity to elevated  $[Ca^{2+}]_c$  that is evident when different human cell lines are compared (see Fig. 8), we analyzed multiple Raji clones that were stably

transfected with either GFP-PL scramblase or with GFP vector alone (Fig. 10). Fig. 10 illustrates that the level of expression of PL scramblase determines plasma membrane sensitivity to intracellular  $\text{Ca}^{2+}$ . The relationship between level of recombinant protein expressed (GFP fluorescence detected in FL1 channel; *abscissa*) and numbers of cells that expose PS after 2 minutes incubation with A23187 (*ordinate*) is plotted for multiple transformed Raji clones. Analysis was gated to include only those cells distinctly positive for GFP fluorescence (FL1 channel), and cell-bound FVa was stained with Tri-color conjugate and detected in FL3 channel (see *Materials and Methods*). Analysis was performed on all cells positive for GFP fluorescence.

Open symbols indicate individual clones stably transformed by transfection with pEGFP-C2; closed symbols indicate individual clones stably transformed with pEGFP-PL scramblase. Data of single experiment, representative of three so performed. These experiments confirm that the capacity of GFP-PL scramblase transformants to mobilize PS to the cell surface generally correlates with the amount of the expressed GFP-PL scramblase fusion protein, whereas this cell response to increased  $[\text{Ca}^{2+}]_i$  is unaffected by cell content of GFP. In addition to confirming the role of PL scramblase in the plasma membrane response to  $[\text{Ca}^{2+}]_i$ , these data suggest that the capacity to mobilize PS to the cell surface and thereby support plasma clotting in activated, injured or

apoptotic cells exposed to elevated  $[Ca^{2+}]_c$  can be altered by changing the level of expression of PL scramblase expressed in the plasma membrane.

#### Discussion

5           These results provide the first evidence that the PL  
scramblase protein identified in the erythrocyte membrane  
and implicated in  $[Ca^{2+}]_c$ -induced remodeling of membrane  
phospholipids actually functions to induce accelerated  
transbilayer movement of plasma membrane phospholipid in  
10 human cells that express this protein. Our results also  
confirm that the level of expression of plasma membrane  
PL scramblase can determine the extent to which PS is  
mobilized to the cell surface upon elevation of  $[Ca^{2+}]_c$ ,  
and suggest that this protein normally functions to  
15 mediate the redistribution of plasma membrane  
phospholipids in response to the entry of calcium into  
the cytosol. Furthermore, these data provide the first  
indication that the movement of PS and other procoagulant  
aminophospholipids from plasma membrane inner leaflet to  
20 the cell surface can be manipulated by selectively  
altering the level of expression of a particular cellular  
protein, either through direct transfection with the PL  
scramblase cDNA, or potentially, by another intervention  
affecting cellular expression of functional PL scramblase  
25 protein.

Whereas these experiments suggest that direct  
activation of plasma membrane PL scramblase is  
responsible for the increased cell surface exposure of PS

that is observed in various activated, injured or apoptotic cells exposed to elevated  $[Ca^{2+}]_c$ , we cannot exclude the possibility that there are other cellular components that contribute to the accelerated movement of PS from inner to outer plasma membrane leaflet under these conditions. In particular, whereas PL scramblase has been shown to mediate the bidirectional movement of PS and other phospholipids between membrane leaflets, it has been suggested that there is also a PS-selective pathway in the platelet plasma membrane, designated "PS floppase", which mediates vectorial movement of PS from inner to outer plasma membrane leaflet (P. Gaffet, et al., Biochemistry 34:6762-6769, 1995). Experimental evidence for the existence of this vectorial and headgroup-selective PS floppase pathway in platelet or other cell membranes remains controversial (R.F.A. Zwaal, et al., supra, 1997; P. Williamson, et al., Biochemistry 31:6355-6360, 1995; C.-P. Chang, et al., J. Biol. Chem. 268:7171-7178, 1993), and awaits identity of a  $[Ca^{2+}]_c$ -activated and PS-selective transporter that is distinct from the plasma membrane PL scramblase found in platelets and erythrocytes, a protein that does not exhibit apparent selectivity for the PS headgroup (J.G. Stout, et al., supra, 1997; P. Comfurius, et al., Biochemistry 35,7631-7634, 1996; F. Bassé, et al., supra, 1996).

In addition to conferring increased sensitivity of the plasma membrane to ionophore-induced elevation of  $[Ca^{2+}]_c$ , we generally observed a higher background of PS

exposure (in absence of ionophore) in those transfected cell clones expressing large amounts of the GFP-PL scramblase fusion protein. This elevated background PS exposure was also observed in the case of untreated HEL, the cell line containing the highest endogenous content of PL scramblase. Although we suspect that this increased background reflects the enhanced sensitivity of the plasma membrane of these cells to any adventitial elevation of  $[Ca^{2+}]_e$  during cell processing for assay, we cannot exclude the possibility that these cells are also inherently more fragile due to the large amounts of PL scramblase that is inserted into the plasma membrane.

While the movement of plasma membrane PS to the cell surface at elevated  $[Ca^{2+}]_e$  can be demonstrated in a variety of cells and tissues (R.F.A. Zwaal, *et al.*, supra, 1997; P. Devaux, supra, 1991), we detect marked differences in the levels of PL scramblase mRNA and protein among different human cell types, which is generally reflected by corresponding differences in sensitivity to this  $[Ca^{2+}]_e$ -induced collapse of plasma membrane PL asymmetry (see Fig. 8, and Q. Zhou, *et al.*, supra, 1997). Although the transcriptional regulation of the PL scramblase gene remains to be determined, it is of interest to note that such cell or tissue-specific differences in PL scramblase expression has the potential to significantly affect the biological properties of the cell. In particular, we note that the content of PL scramblase in human platelet is approximately 10-fold



greater than that of the erythrocyte, which is consistent with the respective PS-mobilizing potential and different roles of these two cells in contributing procoagulant membrane surface for thrombin generation during blood clotting (Q. Zhou, *et al.*, *supra*, 1997). In addition to the relatively high levels of PL scramblase identified in circulating human platelets, this protein was also most abundant in the cell line HEL, whereas only small amounts of this protein (and low PL scramblase activity) was detected for Dami (Fig. 8), two human cancer cell lines exhibiting partial megakaryocytic-like properties. It is also noteworthy that several of the lymphoma-derived cell lines (e.g., Raji, MOLT-4) express considerably reduced levels of PL scramblase, and also show distinctly attenuated PS exposure in response to elevated  $[Ca^{2+}]_c$ , when compared to either peripheral blood leukocytes or to EBV-transforms of normal lymphocytes (Fig. 8). The collapse of plasma membrane phospholipid asymmetry is a relatively early event in apoptosis of lymphocytes and other cells, and the consequent exposure of PS on the cell surface is thought to contribute to phagocytic removal of such cells by scavenger macrophages (V.A. Fadok, *et al.*, *J. Immunol.* 148:2207-2216, 1992; B. Verhoven, *et al.*, *J. Exp. Med.* 182:1597-1601, 1995). It is therefore of interest to consider whether the apparent resistance of certain lymphoma-derived cell lines to such  $[Ca^{2+}]_c$ -induced remodeling of plasma membrane phospholipids might contribute to the proliferative

potential and in vivo survival of these or other transformed cells.

EXAMPLE 4: Induction of Programmed Cell Death is Related to the Cellular Content of PL Scramblase

5 A. Summary

Human cell lines expressing various levels of PL  
scramblase protein were exposed to agents or conditions  
of cell culture known to promote apoptosis (programmed  
cell death). Analysis of these cell lines revealed that  
10 the number of cells induced to undergo apoptosis  
generally correlated with the level of expression of PL  
scramblase protein, with those cell lines expressing high  
levels of PL scramblase being most sensitive to induction  
of apoptosis while cell lines expressing low levels of PL  
15 scramblase being most resistant to induction of  
apoptosis. The human Burkitts B-lymphoma cell line Raji  
was found to express low levels of PL scramblase and to  
be markedly resistant to induction of apoptosis.  
Transfection of Raji cells with plasmid containing cDNA  
20 for PL scramblase resulted in increased sensitivity of  
this cell to the induction of apoptosis, demonstrating  
that expression of PL scramblase in a normally apoptosis-  
resistant cancer cell line promotes programmed death of  
the cancer cell.

25 B. Materials and Methods

Materials. All restriction enzymes were from New  
England BioLabs, Inc. (Beverly, MA). KlenTaq polymerase  
and pEGFP-C2 vector were from CLONTECH Laboratories (Palo  
Alto, CA). Bovine coagulation factor Va (Fva). OPTI-MEM

and geneticin were from Life Technologies (Gaithersburg, MD). Fetal bovine serum, RPMI 1640, Cell Dissociation Solution, Hank's Balanced Salt Solution (HBSS), Protein A Sepharose-CL4B, leupeptin, and BSA were from Sigma Chemical Co. (St. Louis, MO). UltraLink Iodoacetyl resin and SuperSignal ULTRA Chemiluminescence Kit were from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

Antibodies: Anti-GFP: murine monoclonal antibody against green fluorescent protein (GFP) was from CLONTECH Laboratories. Anti-FVa: murine monoclonal antibody V237 reactive against human or bovine factor Va light chain was the generous gift of Dr. Charles T. Esmon (Oklahoma Medical Research Fndn, Oklahoma City, OK). Anti-PL Scramblase-E306-W318: Rabbit antibody raised against the carboxyl terminal peptide sequence E306-W318 of human PL scramblase has previously been described (Q. Zhou, et al., supra, 1997). The IgG fraction was isolated on protein A-Sepharose-CL4B and the peptide-reactive antibody purified by affinity chromatography on peptide [Cys]-ESTGSQEQKSGVW (SEQ ID NO:5) coupled to UltraLink Iodoacetyl resin. The IgG fraction of anti-human FAS ligand was from Clontech (Sunnyvale, CA).

Cell culture: Human cancer cell lines erythroleukemic HEL, promyelocytic leukemia HL-60, chronic myelogenous leukemia K562, lymphoblastic leukemia MOLT-4, acute T-cell leukemia Jurkat, Burkitt's lymphoma Raji, and megakaryocytic DAMI were from American Type

Culture Collection (Rockville, MD) and cultured in RPMI 1640 containing 10% fetal bovine serum. EBV-transformed cell line W9 established from peripheral B-lymphocytes of a normal donor was maintained as previously described (H. Kojima, et al., 1994).

Plasmid Construction: Human PL scramblase cDNA insert was released from plasmid pMAL-C2-PL scramblase (Q. Zhou, et al., supra, 1997) by double cutting with EcoRI and SalI, respectively, and then ligated into pEGFP-C2 vector using the same restriction site. The pEGFP-C2-PL scramblase plasmid was amplified from single clones in *E. coli* strain Top 10, and the orientation and reading frame of the insert confirmed by sequencing on an ABI DNA Sequencer Model 373 Stretch (Perkin Elmer-Applied Biosystems, Foster City, CA) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit.

Transfection of Raji cells with pEGFP-PL scramblase.  $1.6 \times 10^7$  Raji cells were electroporated with 160  $\mu$ g plasmid DNA (pEGFP-C2-PL scramblase or pEGFP-C2) in a total volume of 0.8 ml OPTI-MEM, using Gene Pulse Electroporator (Bio-Rad Laboratories, Hercules, CA) set at 450 V, 500 $^{\circ}$ F. After 48 hours in culture, 1.5 mg/ml geneticin was added to the medium and continuously maintained for 4 weeks. Stable transformants exhibiting GFP fluorescence were sorted by flow cytometry (FACStar, Becton-Dickinson Immunocytometry Systems, San José, CA) using an FL1 sorting gate. The FL1-positive cells were dilutionally cloned in 96 well culture plates. pEGFP-PL

scramblase transformants expressing the 62 kDa GFP-PL  
scramblase fusion protein were identified by Western  
blotting with anti-GFP and with anti-PL-306-W318  
antibodies. Western blotting of pEGFP-C2 transformants  
5 (without insert) confirmed presence of 27 kDa GFP.  
Clones expressing various amounts of GFP-PL scramblase  
were each expanded for functional assay, along with  
comparable GFP-expressing clones serving as controls.

Fluorescence Microscopy. Cell clones transfected  
10 with pEGFP-C2-PL scramblase or pEGFP-C2 were deposited on  
glass microscopy slides using a Cytospin 3 (Shandon,  
Inc., Pittsburgh, PA). Phase contrast and fluorescence  
microscopy was performed with a ZEISS AXIOSKOP microscope  
(Carl Zeiss, Inc., Thornwood, NY) equipped for  
15 epifluorescence, and images were recorded with a MC100  
camera system. The exposure times for photography of  
fluorescence was 80-200 seconds under automatic control  
using Kodak Ektachrome 1600 film.

Western Blot Analysis. Western blotting of GFP and  
20 PL scramblase antigens was performed using  $1.5 \times 10^6$  cells  
per lane. After washing in HBSS, supernatants were  
removed, and the cell pellets extracted with 2% (v/v) NP-  
40 in 5 mM EDTA, 50 mM benzamidine, 50 mM N-ethyl  
maleimide, 1 mM phenylmethylsulfonyl fluoride, 1 mM  
25 leupeptin in HBSS. After removal of insoluble material  
(250,000 x g, 30 minutes, 4°C), the samples were  
denatured at 100°C in 10% (w/v) SDS sample buffer  
containing 2%  $\beta$ -mercaptoethanol. Following SDS-PAGE and

transfer to nitrocellulose, the blocked membrane was incubated with either 10 µg/ml of rabbit anti-PL scramblase-E306-W318, or 1/10,000 dilution of mouse anti-GFP. The blots were developed with the horseradish  
5 peroxidase conjugate of either goat anti-rabbit IgG or goat anti-mouse IgG, respectively, using SuperSignal ULTRA chemiluminescence.

Induction of apoptosis: Apoptosis was induced either by (1) addition of 60 µM etoposide to the cell  
10 culture (2) by removal of fetal bovine serum from the culture medium (serum starvation). Cells were maintained in sterile culture flasks in 37°C incubator under each of these conditions for 72 hours, during which cell aliquots were obtained for assay (times indicated in figure  
15 legends).

Assays for Apoptosis: The induction of apoptosis was detected by (1) egress of PS to the cell surface (2) activation of cellular caspase enzymes (3) cleavage of nuclear DNA. Egress of PS to the cell surface was  
20 measured by the binding of Factor Va (light chain) or by the binding of FITC-annexin V, detected by flow cytometry. Cleavage of nuclear DNA was measured either by a decrease in propidium iodide staining of the nucleus, according to published methods (ref), or by the  
25 measured incorporation of FITC-dUTP into 5'-ends of chromosomal DNA, catalyzed by the enzyme terminal deoxyribose transferase (TUNEL Assay Kit, Clontech) according to the manufacturer's protocol. In each assay,

fluorescence was detected by flow cytometry (FACScan, Becton Dickinson), using the FL1 channel to detect FITC or the FL3 channel to detect propidium iodide staining of the nucleus. Forward and side scatter gates were set to  
5 exclude cell debris.

Measurement of cell surface PS. Exposure of PS on the surface of all cell lines analyzed was detected by the specific binding of coagulation factor Va (light chain) as previously described (P.J. Sims, et al., J. Biol. Chem. 263:18205-18212, 1988; H. Kojima, et al., J. Clin. Invest. 94:2237-2243, 1994). Briefly, cells were washed twice to remove serum proteins and suspended ( $2 \times 10^6$  cells/ml) at 37°C in RPMI 1640 supplemented with 0.1% BSA, 20 mM HEPES, and adjusted to 1.2 mM free  $[Ca^{2+}]$ . At  
10 time=0, A23187 (0 or 2  $\mu$ M final concentration) was added from 1 mM stock solution in DMSO, and at times indicated in figure legends, the reaction was stopped by addition of 6 mM EGTA. PS exposed on the cell surface at each time point was detected by incubating (10 minutes, room  
15 temperature) 50  $\mu$ l of the cell suspension with 10  $\mu$ g/ml FVa, followed by 10  $\mu$ g/ml anti-FVa, to detect the cell-bound FVa light chain. After staining with 10  $\mu$ g/ml Tri-Color conjugated goat anti-mouse IgG (CALTAG  
20 Laboratories, Burlingame, CA), single-cell fluorescence was quantitated by flow cytometry (FL3 channel, FACScan, Becton Dickinson Immunocytometry Systems). Use of Tri-Color conjugate to detect cell-bound FVa enabled  
25 simultaneous measurement of cell-associated GFP

fluorescence in cell lines transformed with the pEGFP-C2 expression plasmid (fluorescence of GFP detected in FL1 channel). In experiments in which cell lysis was monitored by uptake of propidium iodide, cells were stained for bound FVa with FITC-conjugate of goat anti-mouse IgG (FL1 channel) substituting for Tri-Color conjugate, and propidium iodide was detected in FL3 channel. Propidium iodide (0.5  $\mu$ g/ml) was added immediately before dilution for flow cytometry.

10 C. Results

Role of PL scramblase in PS mobilization under conditions of induced apoptosis. As it appeared that the cellular content of PL scramblase determines the sensitivity of the plasma membrane to accelerated transbilayer migration of PS induced through elevation in cytoplasmic  $Ca^{2+}$  (see Example 3, supra), we considered whether the expression of this protein might also determine the extent to which PS is mobilized to cell surfaces under conditions of programmed cell death. As previously reported, the extent to which PS is exposed at cell surfaces when cells are subjected to conditions that ultimately result in their apoptotic death, vary widely between different cell lines, suggesting that the reorganization of plasma membrane phospholipids during programmed cell death is a regulated response inherent to particular cell lines (T. Frey, et al., J. Cytometry 28:253-263, 1997). In particular, both Raji and HL60 have recently been noted to be relatively resistant to



apoptotic induction of cell surface PS (D.L. Bratton, et al., supra, 1997; T. Frey, supra, 1997), consistent with the resistance we observe in the response of these cell lines to a direct elevation of  $[Ca^{2+}]_i$ . (See Example 3, above). We therefore compared PS exposure in the B-lymphoblast cell lines Raji (low level PL scramblase expression) and W9 (high level PL scramblase expression) under conditions of serum starvation which has been shown to induce apoptotic death in human B-lymphoblasts (T. Frey, supra, 1997).

Fig. 14 illustrates the role of PL scramblase in PS exposure during apoptosis of B-lymphoblasts. Referring to Fig. 14, PS exposure (measured with Fva light chain) in human B-lymphoblast cell lines during apoptosis induced by one day incubation in reduced serum (1% fetal bovine serum; *solid bars*). Open bars represent identically matched controls maintained continuously in 10% serum. Panel A compares the Raji (low endogenous PL scramblase expression) and W9 (high PL scramblase expression) cell lines. Panel B compares individual Raji cell clones that were stably transfected with either pEGFP-C2 (GFP) or with pEGFP-PL scramblase (GFP-PLS) and then induced to apoptosis under the conditions described for Panel A. Error bars denote mean  $\pm$  SD ( $s=4$ ). Significance of the increase in PS exposure due to serum starvation by paired t-test, \* $p<0.001$  (W9); \*\* $<0.02$  (GFP-PLS).

As illustrated by Fig. 14 (panel A), when each cell line was incubated under conditions inducing apoptosis, PS exposure observed for W9 was always substantially elevated above that observed for Raji, consistent with both the greater quantity of PL scramblase protein expressed in the W9 cell line and this cell's increased sensitivity to PS mobilization through  $\text{Ca}^{2+}$  ionophore. This increased activity of the PL scramblase pathway in W9 versus Raji was also apparent when apoptosis was induced in these cells by exposure to etoposide (data not shown). Furthermore, we observed that the extent to which PS was mobilized to the surface of Raji cells undergoing apoptosis was increased through prior transfection of these cells to express the GFP-PL scramblase fusion protein (Fig. 14, panel B). These data suggest that the extent to which plasma membrane PS becomes exposed at the cell surface during induced apoptosis of lymphoblasts and other cell lines is directly related to the cellular content of PL scramblase protein.

Expression of PL scramblase promotes intracellular events that result in apoptotic cell death. In order to determine whether the level of expression of PL scramblase alters the capacity of a cell to respond to an external signal to undergo programmed cell death, we used the GFP-PL scramblase-transfected Raji cell lines to monitor fragmentation of nuclear DNA in response to known apoptotic stimuli. In these experiments, the transfected

cells were induced to apoptosis by either culture under serum starvation conditions or by challenge with etoposide. Nuclear degradation was monitored by TUNEL assay (Clontech) and data analyzed as per cent of all  
5 cells induced to DNA fragmentation.

Fig. 15 illustrates transfection with cDNA encoding PL scramblase increases apoptotic response of human B-lymphoma line Raji. Raji cell lines were stably transfected with plasmid to express either GFP-PL  
10 scramblase fusion protein (GFP-PLS; hatched bars) or pGFP vector as control (GFP; solid bars) and analyzed under conditions of induced apoptosis in cell culture. Also shown are data for non-transfected parental cell line Raji (Raji; open bars). Each cell line was incubated  
15 overnight under either serum-starved conditions (0% serum) or in presence of 60  $\mu$ M etoposide (Etoposide). Control refers to identical cells maintained under normal culture conditions of 10% fetal bovine serum. Apoptosis was assessed by degradation of nuclear DNA, as measured  
20 by TUNEL assay (Clontech) and is expressed as percent of total cells induced to undergo nuclear fragmentation. Data of a single experiment, representative of at least five similar experiments so performed.

As shown in Fig. 15, these experiments revealed that  
25 whereas untransfected Raji cells or GFP-vector transfected Raji cell lines were highly resistant to induction of apoptosis, the Raji cell lines expressing the GFP-PL scramblase fusion protein were readily induced

to apoptosis, culminating in fragmentation of nuclear DNA. Consistent with these data, we also observed that the cell lines W9 and Jurkat (expressing high levels of endogenous PL scramblase) were markedly more sensitive to  
5 induction of apoptosis than the cell line Raji (which normal expresses little PL scramblase (data not shown)). In addition to suggesting that the sensitivity of a cell to induction of programmed cell death can be related to the level of expression of PL scramblase, these  
10 experiments establish that a transformed tumor cell line (the human Burkitts lymphoma cell line, Raji) that is normally resistant to apoptosis, can be altered through transfection with cDNA encoding PL scramblase to be sensitive to induction of programmed cell death.

## CLAIMS

We claim:

1. A method for extending the viability of mammalian cells or tissues comprising the step of inhibiting the expression of native PL scramblase within the cell or tissue.

2. The method of claim 1 wherein the inhibition is *in vitro*.

3. The method of claim 1 wherein the inhibition is *in vivo*.

4. The method of claim 1 wherein the mammalian cell or tissue is selected from the group consisting of a blood cell stored for transfusion, a hematopoietic stem cell, an endothelial cell, a pancreatic islet cell, a  
5 blood vessel, a skin or other tissue graft, or solid organ for transplantation.

5. The method of claim 1 wherein the inhibition is via a PL scramblase antisense RNA molecule.

6. The method of claim 1 wherein the cell or tissue is a human cell or tissue.

7. The method of claim 1 wherein expression is inhibited via a mutant or truncated form of PL scramblase protein that is inhibitory to endogenous PL scramblase protein.

8. The method of claim 7 wherein the truncated form of the protein is the translation product of an alternatively spliced PL scramblase mRNA.

9. The method of claim 7 wherein the mutant PL scramblase contains one or more non-conservative substitution of residues in the Asp<sup>273</sup>-to-Asp<sup>284</sup> region of PL scramblase polypeptide.

10. The method of claim 1 wherein the function of cell PL Scramblase is inhibited by preventing fatty acylation of the nascent PL scramblase polypeptide.

11. The method of claim 1 wherein the function of cell PL scramblase is inhibited by deacylation of the mature PL scramblase protein.

12. a method of decreasing the viability, metastatic or invasive potential of cancer cells, cancerous tissue, or viral-infected cell by causing increased expression or activity of PL scramblase protein  
5 within the cell or tissue.

13. The method of claim 12 wherein the cancer cell represents a leukemia, lymphoma, carcinoma, adenoma, sarcoma, or other transformed cell type with oncogenic, metastatic, or tumorigenic potential.

14. The method of claim 12 wherein the cell is infected with a human retrovirus, human leukemia virus, human adenovirus.

15. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell is transfected to express an exogenous PL scramblase coding sequence.

16. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell expresses exogenous PL scramblase protein.

17. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell is within a human patient.

18. The method of claim 12 wherein the increased expression of PL scramblase protein is achieved by decreasing expression of alternatively spliced transcripts of PL scramblase within the cancer cell,  
5 cancerous tissue or viral-infected cell.

19. A method of determining the status of a patient's cancer comprising the steps of analyzing the level of PL scramblase RNA or PL scramblase protein within cancer cells and correlating the level with a  
5 standard curve.

20. A method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue comprising the step of analyzing the amount of PL scramblase RNA or protein within a patient's  
5 cell sample.

21. The methods of claims 19 or 20 wherein the level of PL scramblase RNA is measured by *in situ* hybridization or Northern blotting with PL scramblase cDNA or by the polymerase chain reaction using  
5 oligonucleotide primers based on sequence of human PL scramblase cDNA.

22. The method of claims 19 or 20 wherein the level of PL scramblase protein is measured by immunofluorescence, flow cytometry, Western blotting, radioimmunoassay, or by ELISA assay using antibody that  
5 specifically binds PL scramblase protein.

23. A method for inducing apoptosis in a cell comprising the step of increasing expression of PL scramblase within the cell.



24. The method of claim 23 wherein the increase in PL scramblase expression is by activating the promoter of an endogenous PL scramblase gene.

25. The method of claim 23 wherein the increase in PL scramblase expression is by transfection of the cell with cDNA encoding PL scramblase.

26. The method of claim 23 wherein the cell is part of a tissue.

27. A method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue comprising the step of analyzing the sequence of a patient's PL scramblase gene, RNA or protein for the presence or absence of mutations.

28. The method of claim 27 wherein the analysis comprises techniques selected from the group consisting of RFLP, sequencing by RT-PCR, Northern blotting, Western blotting, electrophoretic gels, protease digestion and other techniques designed to analyze DNA, RNA or protein sequence.

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1  cgcgccgcgtcgaccgaaaccaggagccgcggtgttggcgcaaaaggttactcccagac
61  cctttccggctgacttctgagaaggttgccgagcagctgtgccgacagctctagagcg
121  cagaaggaagccatcgccctggcccggtctctggaccttgctcgtcgggagcgga
181  aacagcgcagccagagaactgtttaatcatggacaaacaaactcacagatgaatgct
      M D K Q N S Q M N A
241  tctcaccggaaacaaacttgccagttgggtatcctcctcagtatccaccgacagcattc
      S H P E T N L P V G Y P P Q Y P P T A F
301  caaggacctccaggatagtggtaccctgggccccagggtcagctaccacccccacca
      Q G P P G Y S G Y P G P Q V S Y P P P P
361  gccggcattcaggtcctggcccagctggcttctcctgtcccaaatcagccagtgataat
      A G H S G P G P A G F P V P N Q P V Y N
421  cagccagtataataatcagccagttggagctgcaggggtaccatggatgccagcgcacag
      Q P V Y N Q P V G A A G V P W M P A P Q
481  cctccattaaactgtccacctggattagaataatttaagtcagatagatcagatactgatt
      P P L N C P P G L E Y L S Q I D Q I L I
541  catcagcaaatgaacttctggaagttttaacaggttttgaaactaataacaaatatgaa
      H Q Q I E L L E V L T G F E T N N K Y E
601  attaagaacagcttggacagagggtttacttgcagcgggaagatactgattgctgtacc
      I K N S F G Q R V Y F A A E D T D C C T
661  cgaattgctgtggccatctagaccttttaccttgaggattattgataatatgggtcaa
151  R N C C G P S R P F T* L R I I D N M G Q

```

FIG. 1A

2/20

721 gaagtcataactctggagagaccactaagatgtagcagctgttggtccctgctgcctt  
171 E V I T L E R P L R C S S C C P C C L  
781 caggagatagaatccaagctcctcctggtgtaccaataggttatgttattcagacttgg  
191 Q E I E I Q A P P G V P I G Y V I Q T W  
841 caccatgtctaccaaaagtttacaattcaaatgagaaaaagagagatgtactaaaaata  
211 H P C L P K F T I Q N E K R E D V L K I  
901 agtggccatgtgtgtgtgcagctgttggtggagatgttgattttgagattaaatctctt  
231 S G P C V V C S C C G D V D F E I K S L  
961 gatgaacagtgtgtgttggaataattccaagcactggactggaattttgagagaggca  
251 D E Q C V V G K I S K H W T G I L R E A  
1021 ttacagacgctgataaactttggaatccagttcccttttagaccttgatgttaaatgaaa  
271 F T D A D N F G I Q F P L D L D V K M K  
1081 gctgtaatgattggtgcctgtttcctcattgacttcatgtttttgaaagcactggcagc  
291 A V M I G A C F L I D F M F E S T G S  
1141 caggaacaaaaatcaggagtgtggtagtggaattagtgaaagtctcctcaggaaatctgaa  
311 Q E Q K S G V W -  
1201 gtctgtatattgattgagactatctaaactcatacctgtatgaattaagctgtaaggcct  
1261 gtagctctggtgtatacttttgccttttcaaattatagtttatcttctgtataactgatt  
1321 tataaagggttttgtacattttttaatactcattgtcaatttgagaaaaaggacatatga  
1381 gtttttgcatttattaatgaaacttcctttgaaaaaactgcttttaaaaaaagtcgacgcg  
1441 gccgc

FIG. 1B

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1 tctaaagactcaggaacaaacctaattgcctcaaaagttcaggtgcttttctccctg  
 61 actttagtctagtgagtagtgcagcacctatgcctttctgagaggagtctggagagctg  
 121 agtcgctgctggtgtaggattctaggaattcgcctcacttgagctgcatgagaaaaga  
 181 aaggcttgcaaatggaggctcctcgctcaggaacatacttgccagctgggtatgccctc  
                   M E A P R S G T Y L P A G Y A P Q  
 241 agtatacctcagcagcagtcctcaaggacctccagagcatactggacgccccacattccaga  
 18 Y P P A A V Q G P P E H T G R P T F Q T  
 301 ctaactaccaagttccccagtgctggttatccaggacctcaggtcaggtacacagctctcaa  
 38 N Y Q V P Q S G Y P G P Q A S Y T V S T  
 361 catctggacatgaagggttatgctgctacacgggttcctattcaaaataatcagactatag  
 58 S G H E G Y A A T R L P I Q N N Q T I V  
 421 tccttgcaaacactcagtggtgagtcagcaccaccacctattctgaactgcccacctgggc  
 78 L A N T Q W M P A P P P I L N C P P G L  
 481 tagaatacttaaatcagatagatcagcttctgattcatcagcaagttgaacttctagaag  
 98 E Y L N Q I D Q L L I H Q Q V E L L E V  
 541 tcttaacaggctttgaaacaaataacaaatttgaatacaagaacagcctcgggcagatgg  
 118 L T G F E T N N K F E I K N S L G Q M V  
 601 tttatgttcagtggaagatactgactgctgtactcgaaattgctgtgaagcgtctagac  
 138 Y V A V E D T D C C T R N C C E A S R P  
 661 cttcaccttaagaatcctggatcatctggtggccaagaagtcactctggagcgacctc  
 158 F T L R I L D H L G Q E V M T L E R P L  
 721 tgagatgcagtagctgctgtcttccccctgctgcctccaggagatagaaatccaggctcctc  
 178 R C S S C C F P C C L Q E I E I Q A P P  
 781 cgggggtgccaatagggttatgtgactcagacctggcaccctgtctgccaaagctcactc  
 198 G V P I G Y V T Q T W H P C L P K L T L

FIG.

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841  ttcagaacgacagaggagaatgttctaaaagtagttggtccatgtgttgcacacct
218      Q N D K R E N V L K V V G P C V A C T C
901  gctgttcagatatgtgactttgagatcaagtctcttgatgaagtgactagaattggtaaga
238      C S D I D F E I K S L D E V T R I G K I
961  tcaccaagcagtggtctggttggtgaaagaggccttcacggattcggataactttggga
258      T K Q W S G C V K E A F T D S D N F G I
1021  tccaattcccgtagacctggagggtgaagatgaaagctgtgacgcttggtgcttgcctcc
278      Q F P L D L E V K M K A V T L G A C F L
1081  tcatagattacatgttttttgaggctgtgagtaggaacagaaatccgacctgcagtagg
298      I D Y M F F E G C E -
1141  aatcaatgaaagaggacagagaagatctgaagtctacacaaggagatcatatgattgaga
1201  gacctggggctttttgatttcttcattgaaatttctcagaatcaagctgttatcatgaa
1261  gcatagtatgtaacattttgggttttcaaatggtagtttatctttacattattgggaatag
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1441  ataactcgggatttatttttcatctctttgttcaatttaaaccttgttagtgctgatttta
1501  ttataaaaattgtactttactatcaaacctagtttagtttatcttacagaaaatcctccta
1561  ttattttgaaattacatatatttttgaaagcttttttaaaagatactattgcctgggaaatc
1621  ta

```

FIG. 1D

5/20

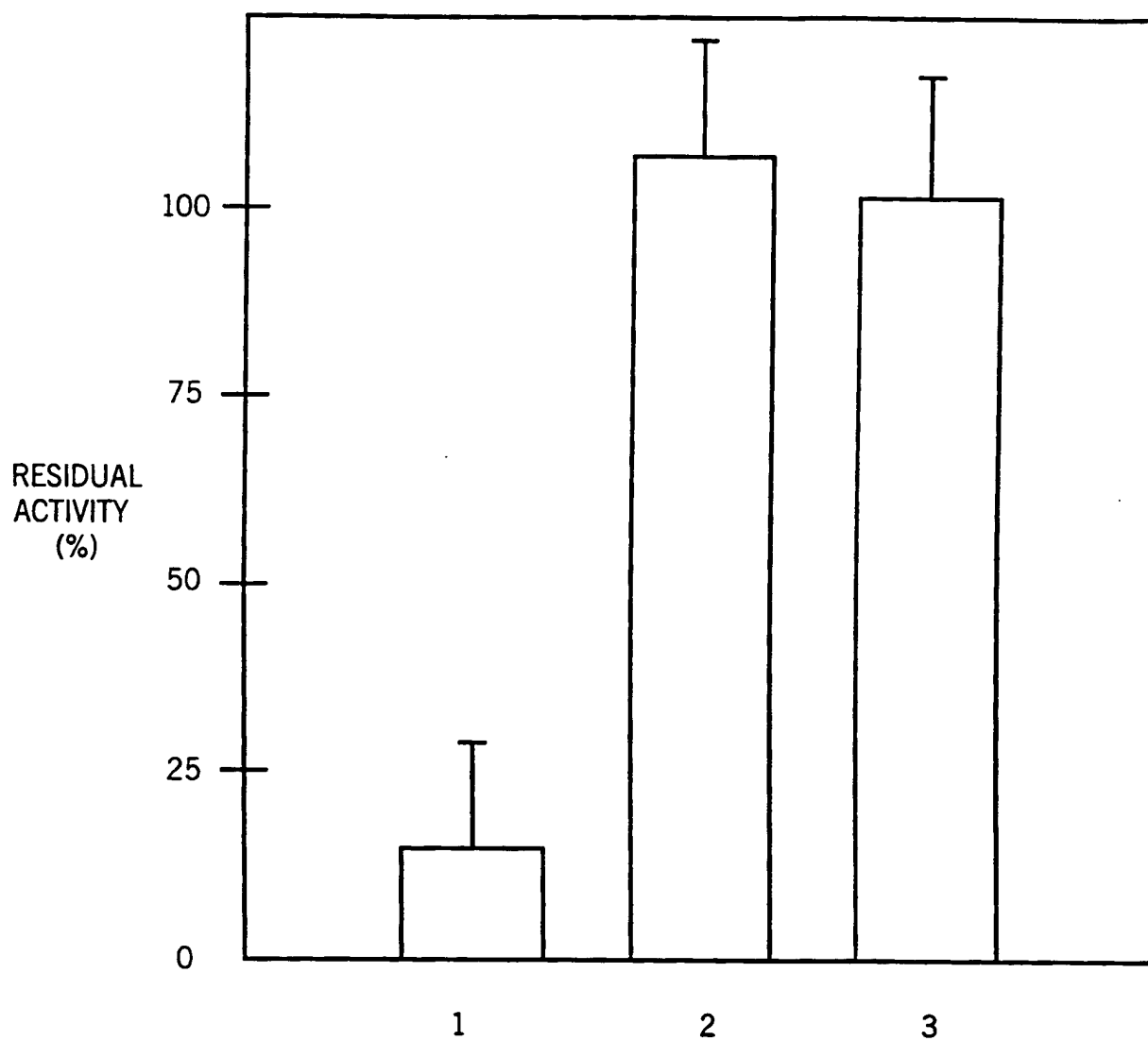


FIG. 2

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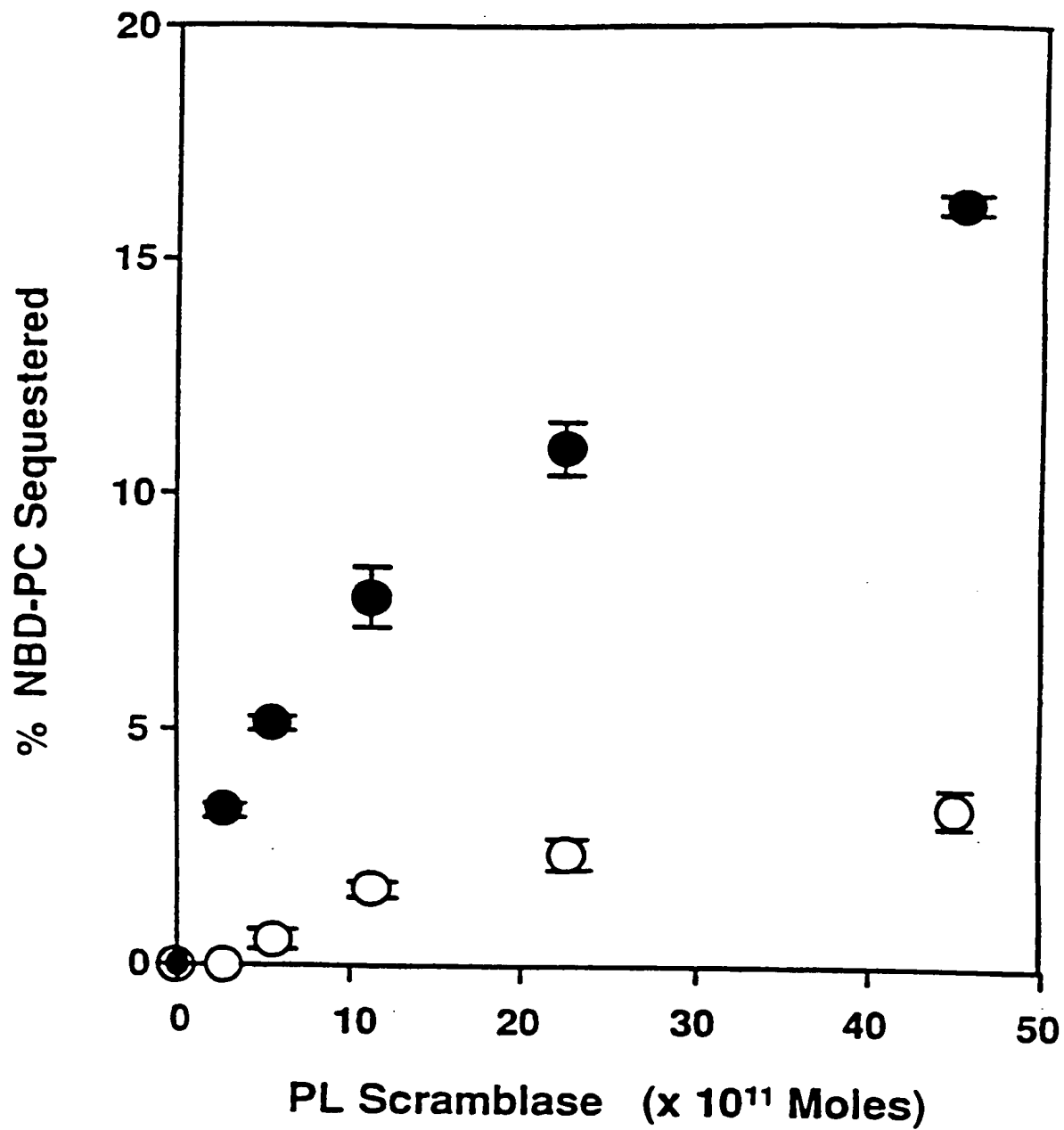


FIG. 3

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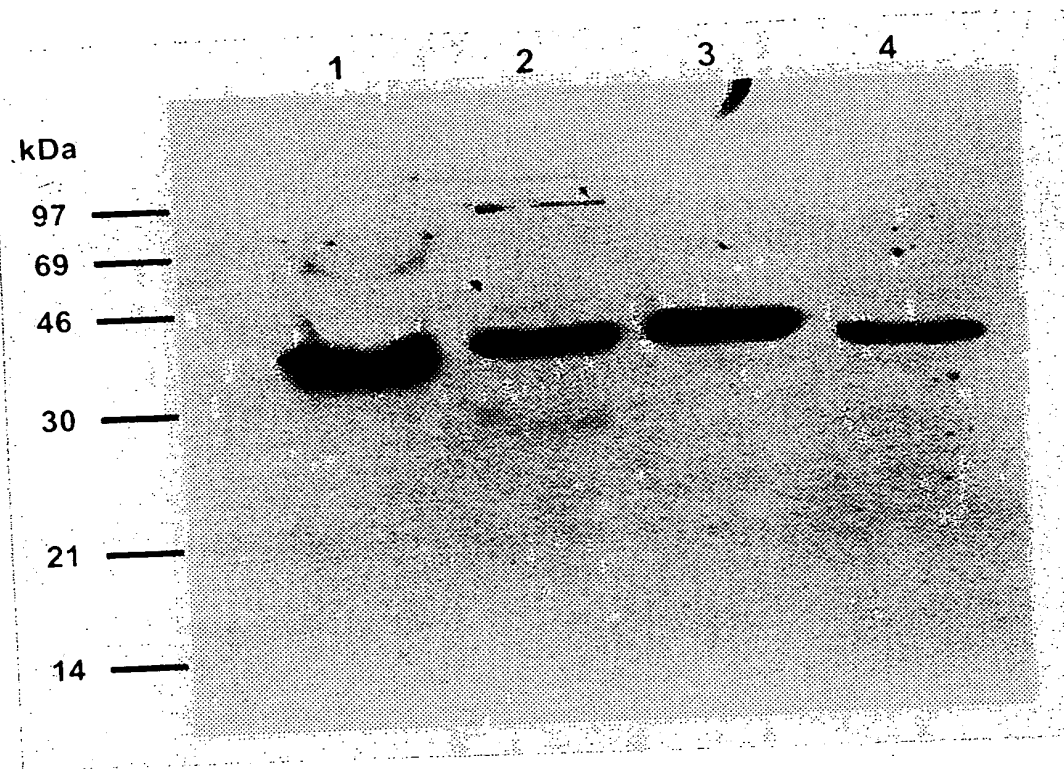


FIG. 4



MUR	10	20	30	40	50
	MEAPRSGTYLPAGYAPQYPPAAVQGPPEHTGRPTFQ	TNYQVPQSGYPGPQASY			
	.....	.....	.....	.....	.....
HUM	MDKQNSQMNASHPETNLPVGYPPQYPP	TA	FQGP	PGYSGYPGPQVSYPPPPAGHSGGPA-	
	10	20	30	40	50
MUR	60	70	80	90	100
	TVSTSGHEGYAATRLPIQNNQ	TIVLANTQWMPAPPPILNCPPGLEYNQIDQLLIHQQVE			
	.....	.....	.....	.....	.....
HUM	GFPVPNQPVYNQ--PV-YNQPVGAAGVPWMPAPQPP	PLNCPPGLEYSQIDQILIHQQIE			
	60	70	80	90	100
					110
MUR	120	130	140	150	160
	LLEVL	TGFETNNKFEIKNSLQGMVYVAVED	TDCC	TRNCCEASRPFTLRILDHLGQEVMTL	
	.....	.....	.....	.....	.....
HUM	LLEVL	TGFETNNKYEIKNSFGQRVYFAAED	TDCC	TRNC	CGPSRPFTLRIDNMGQEVITL
	120	130	140	150	160
					170

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**FIG. 5A**

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	240	250	260	270	280	290
MUR	ACTCCSDIDFEIKSLDEVTRIGKITKQWSGCVKEAFTDSDNFGIQFPPLDLEVKMKA	VTLG				
	.....	.....	.....	.....	.....	..
HUM	VCSCCGDVDFEIKSLDEQC	VVGKISKHWTGILREAF	TDADNFGIQFPPLD	VKMKA	VMI	
	240	250	260	270	280	290

MUR	300	<u>ACFLIDYMFEGCE</u>
		: : : : : :
HUM		<u>ACFLIDFMFFESTGSQEKGW</u>
	300	310

**FIG. 5B**

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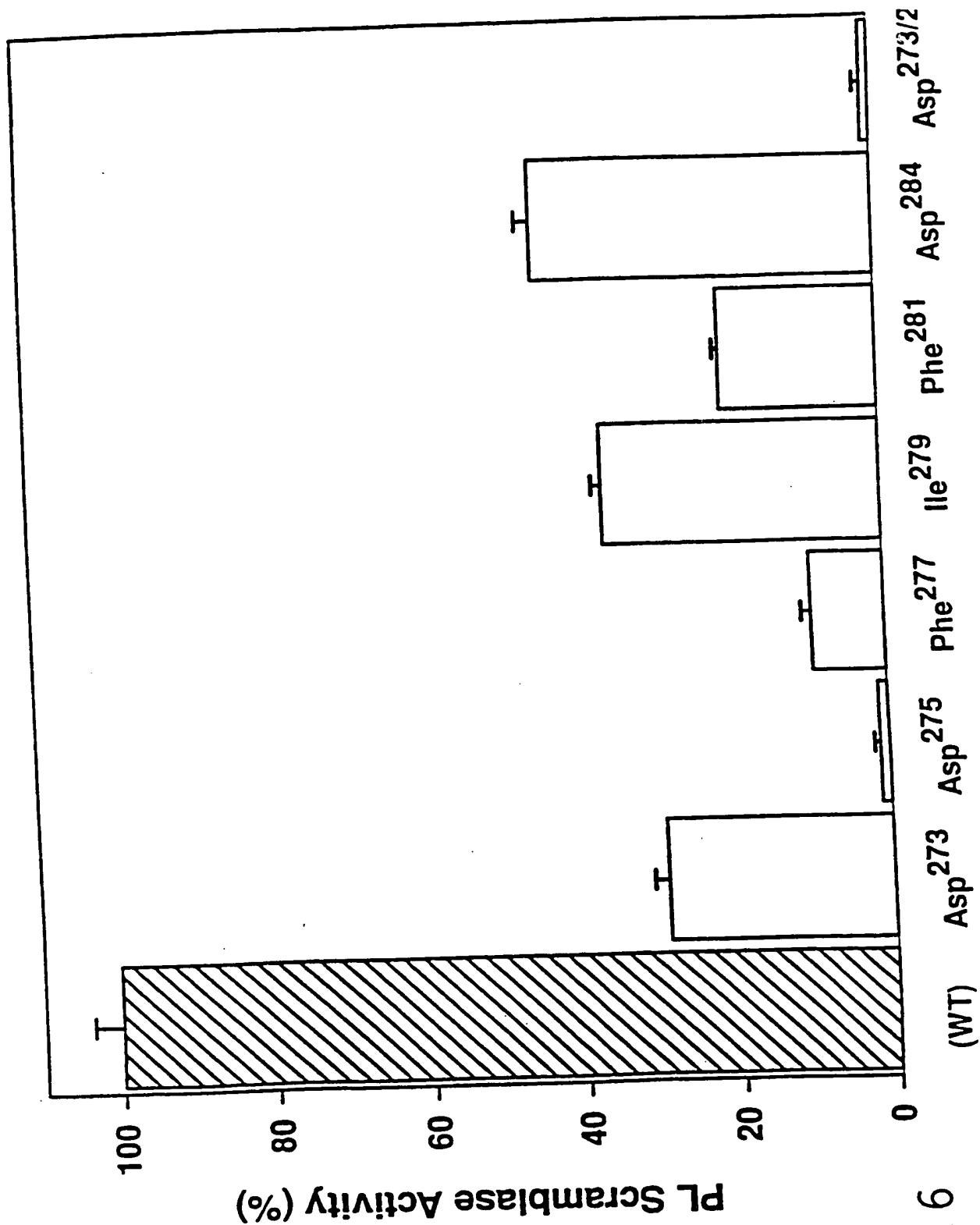


FIG. 6

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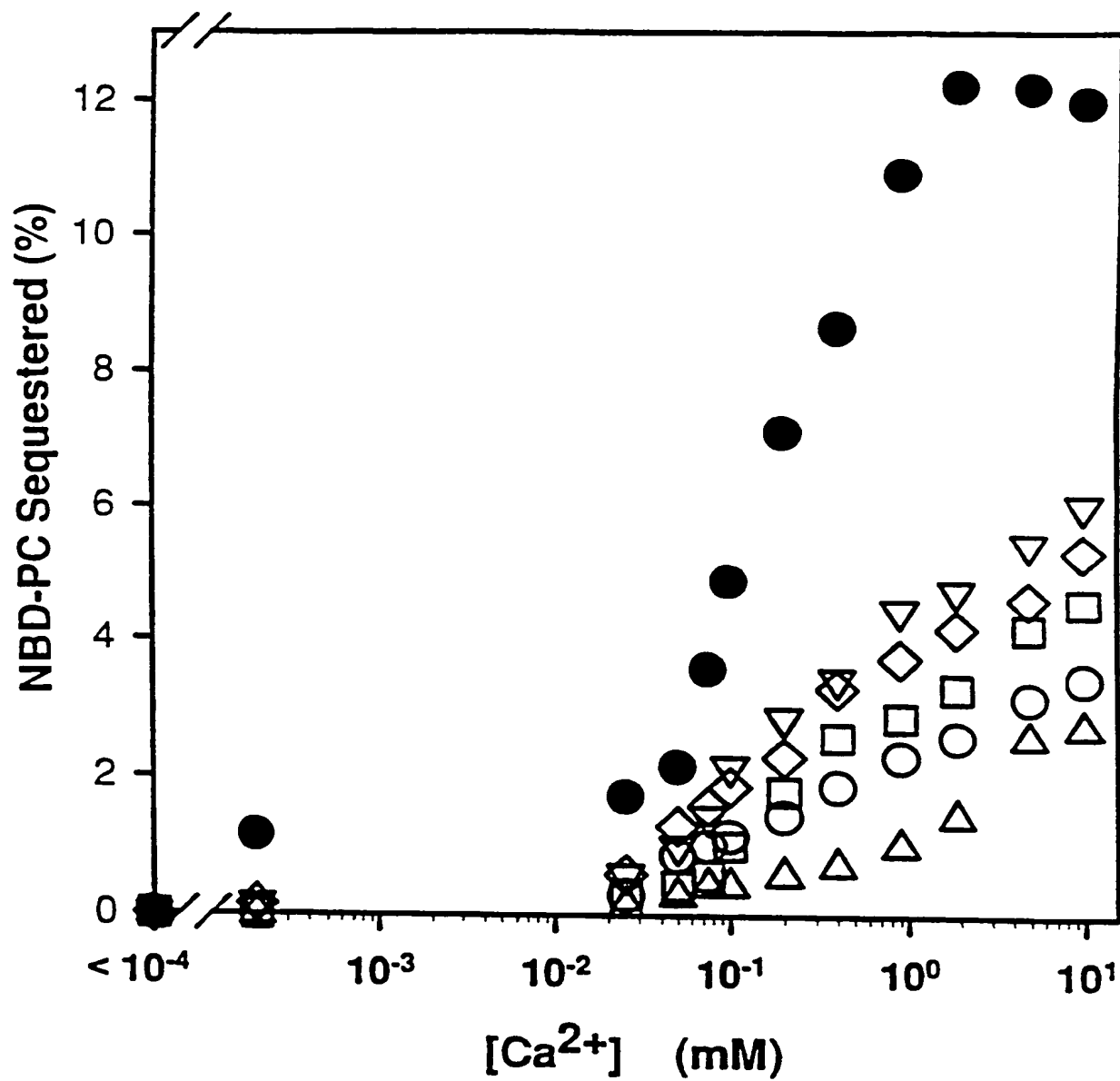


FIG. 7

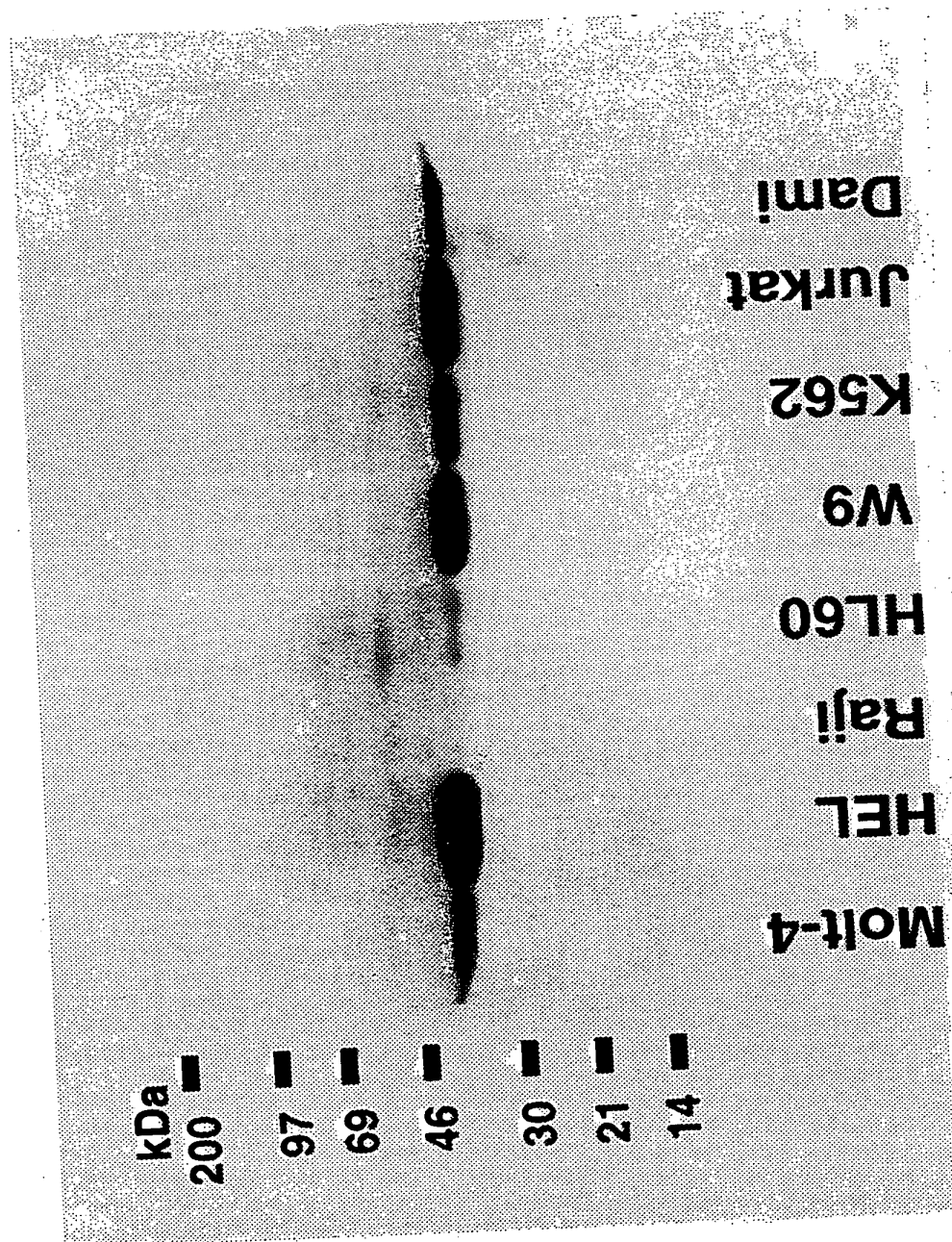


FIG. 8A

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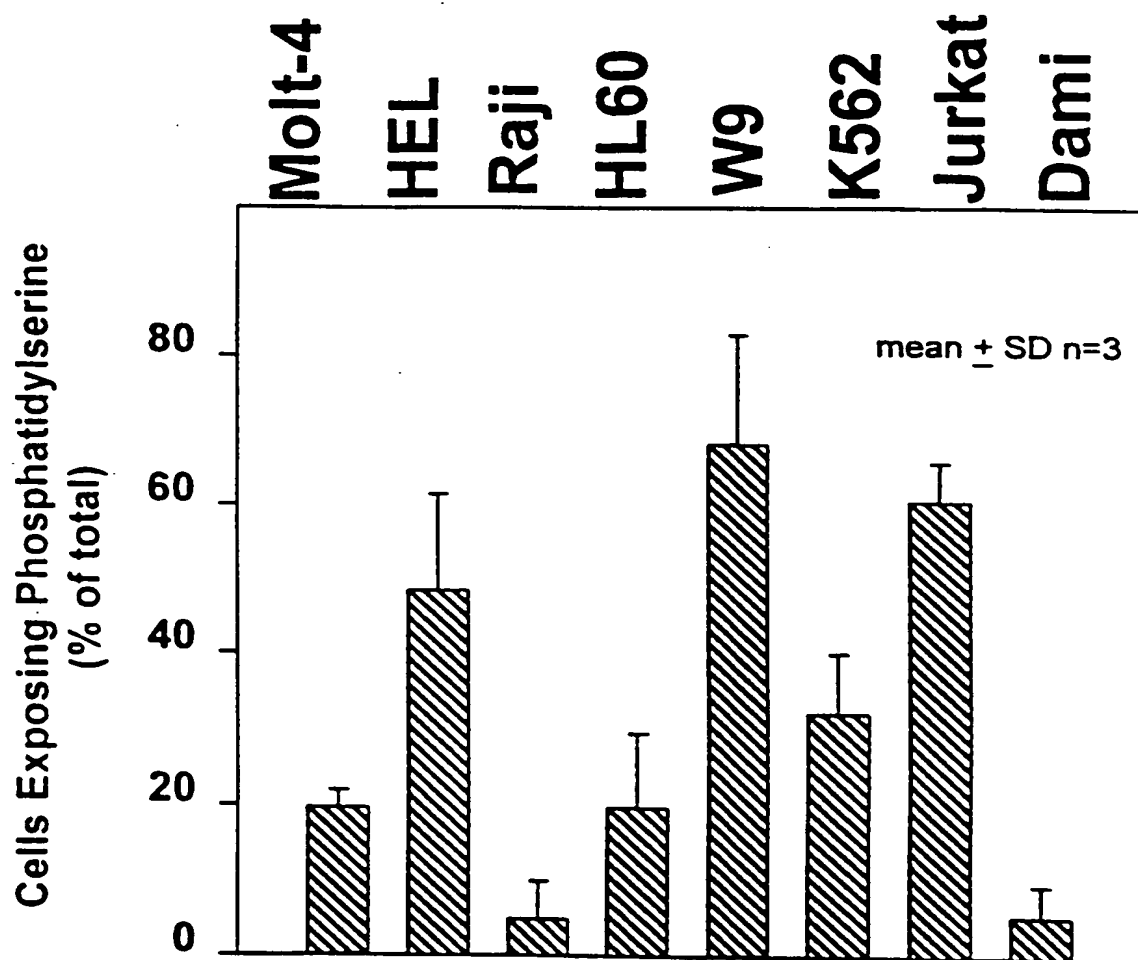


FIG. 8B

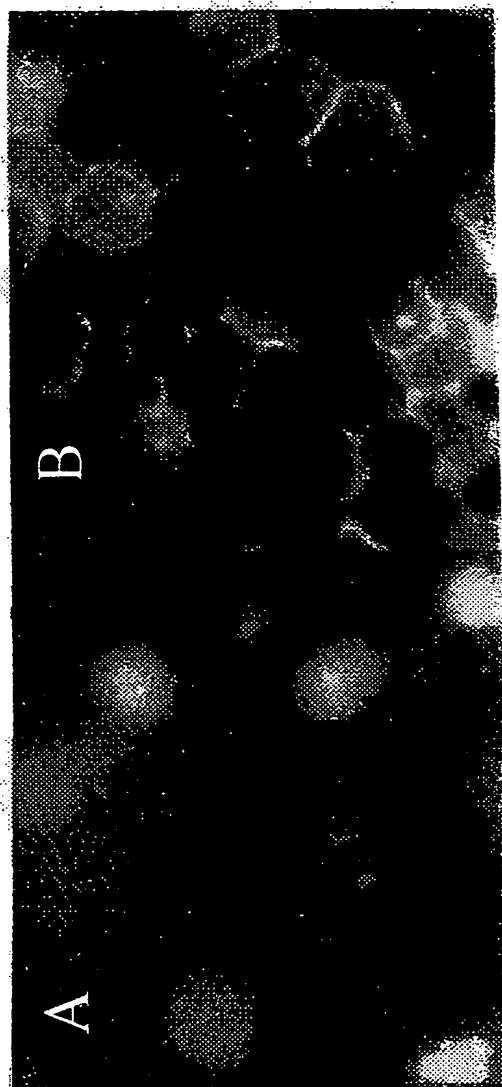


FIG. 9

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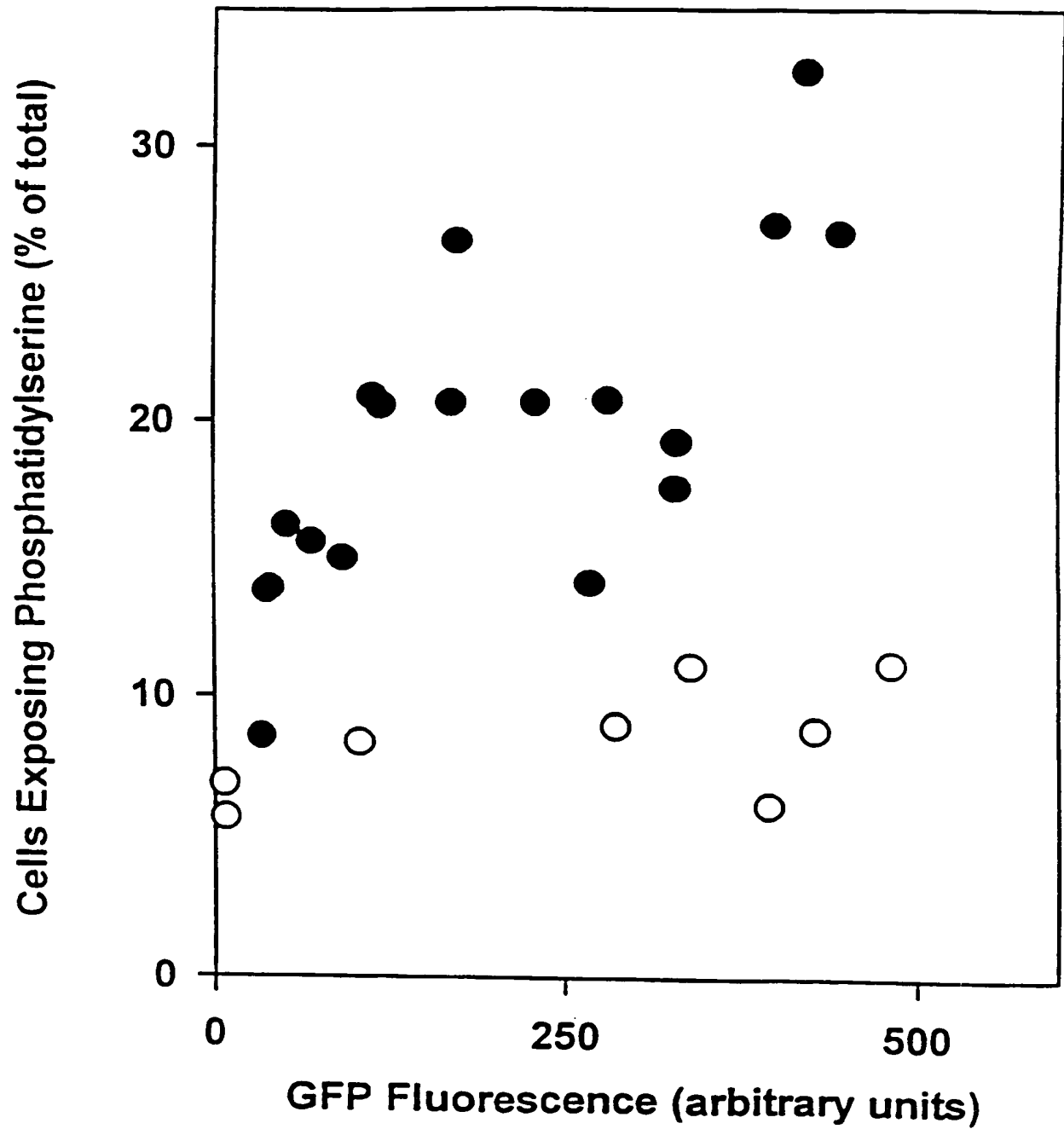
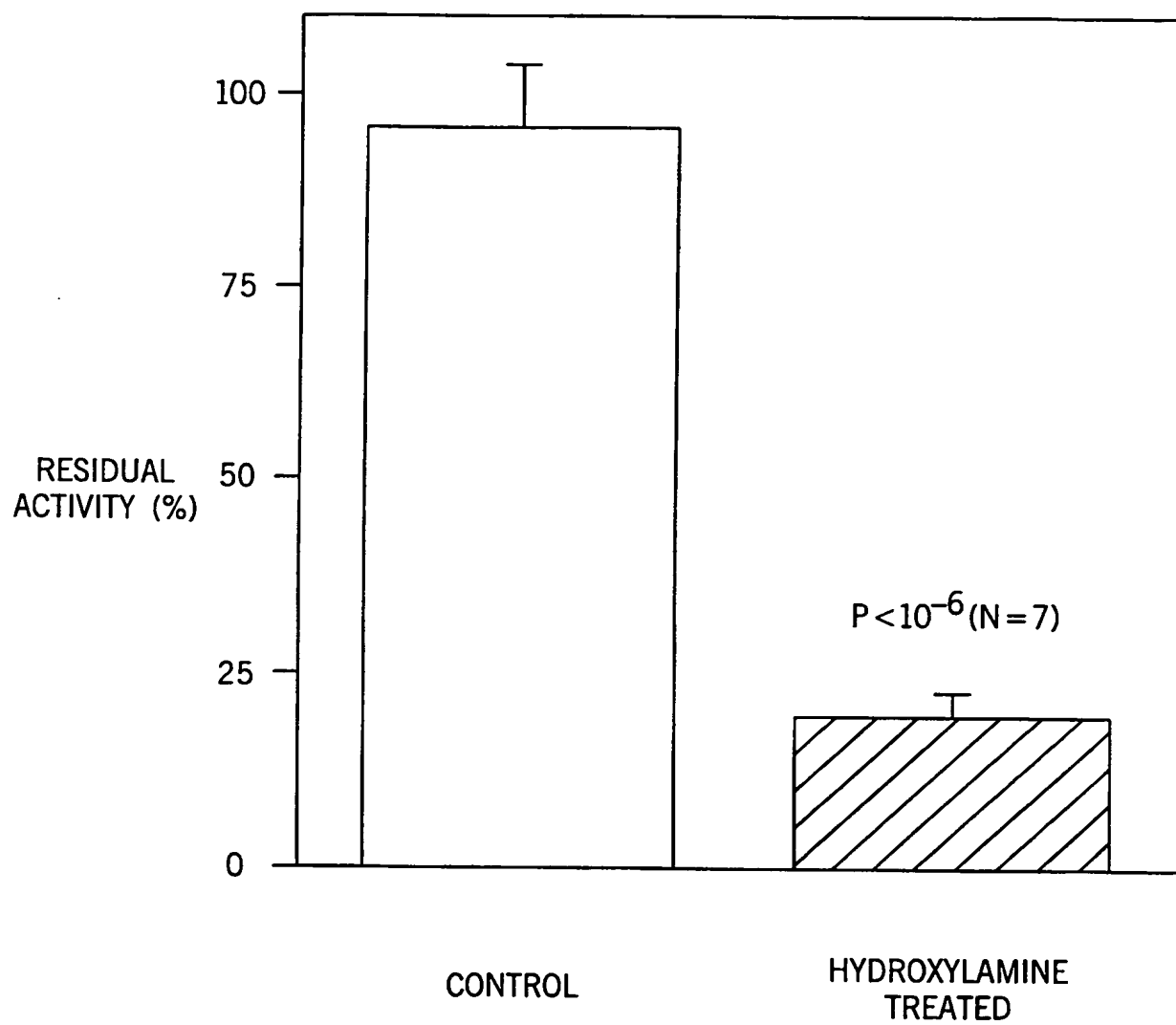


FIG. 10



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FIG. 11



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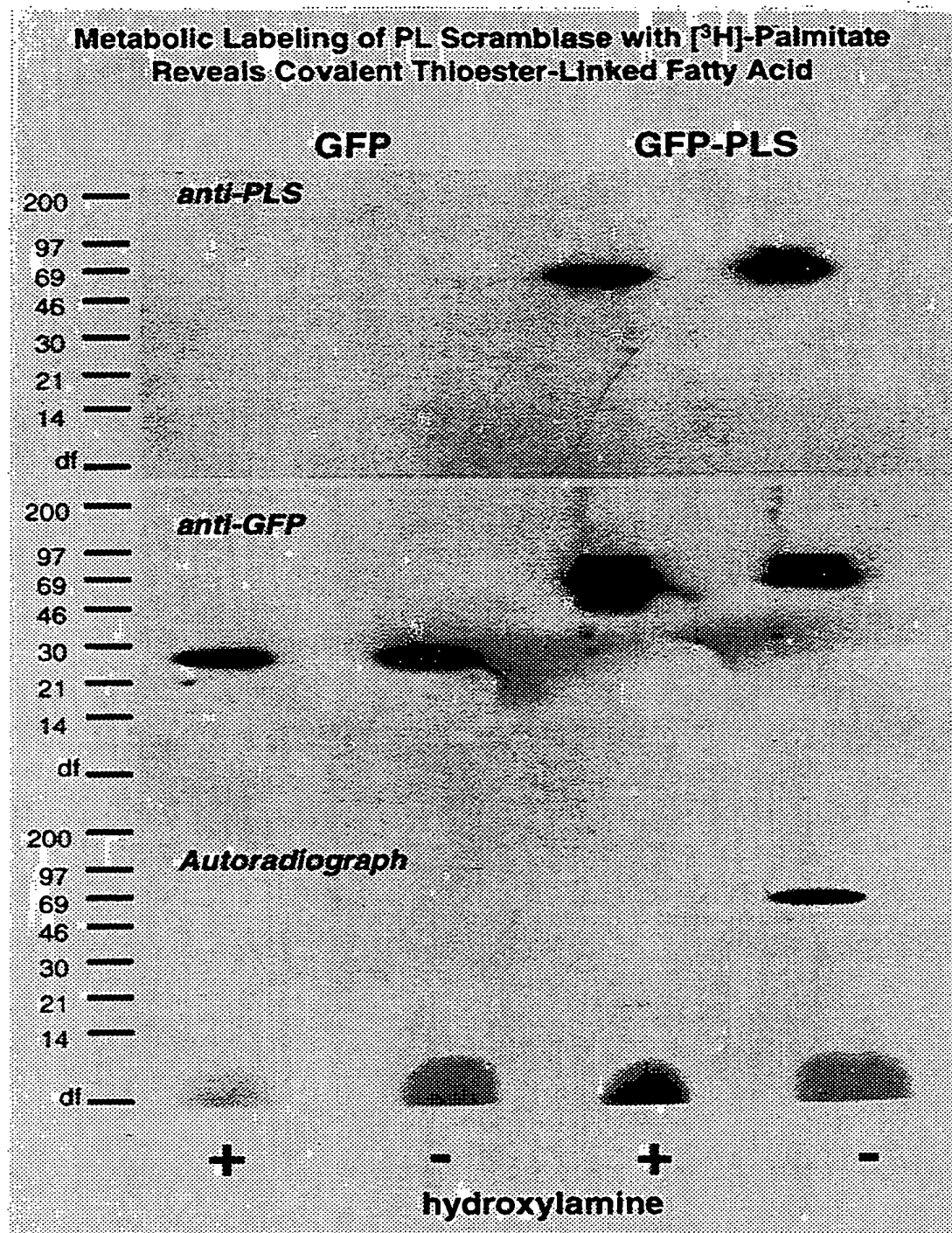


FIG. 12

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**TLC Analysis of [<sup>3</sup>H]-Fatty Acid From  
Hydroxylamine-Treated PL Scramblase**

SF —

OR —

palmitate

+

-

palmitate

FIG. 13

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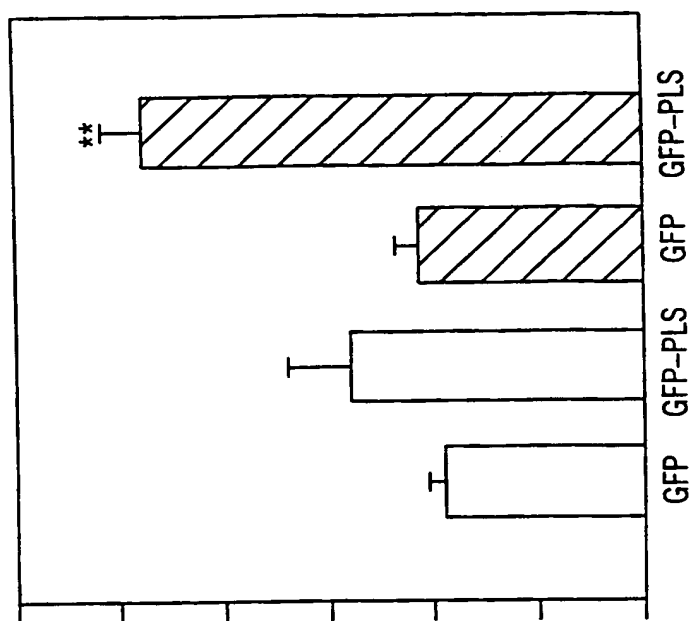


FIG. 14B

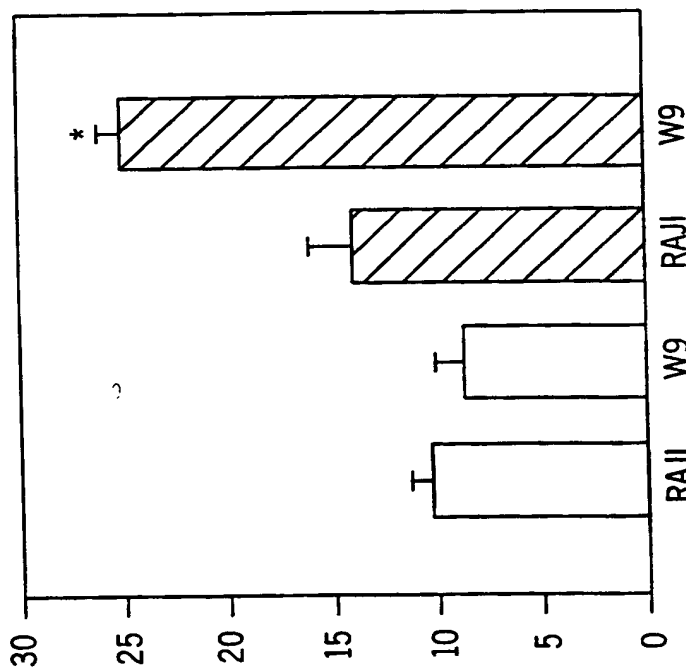


FIG. 14A

CELLS EXPOSING  
PHOSPHATIDYLSERINE  
(% OF TOTAL)

20/20

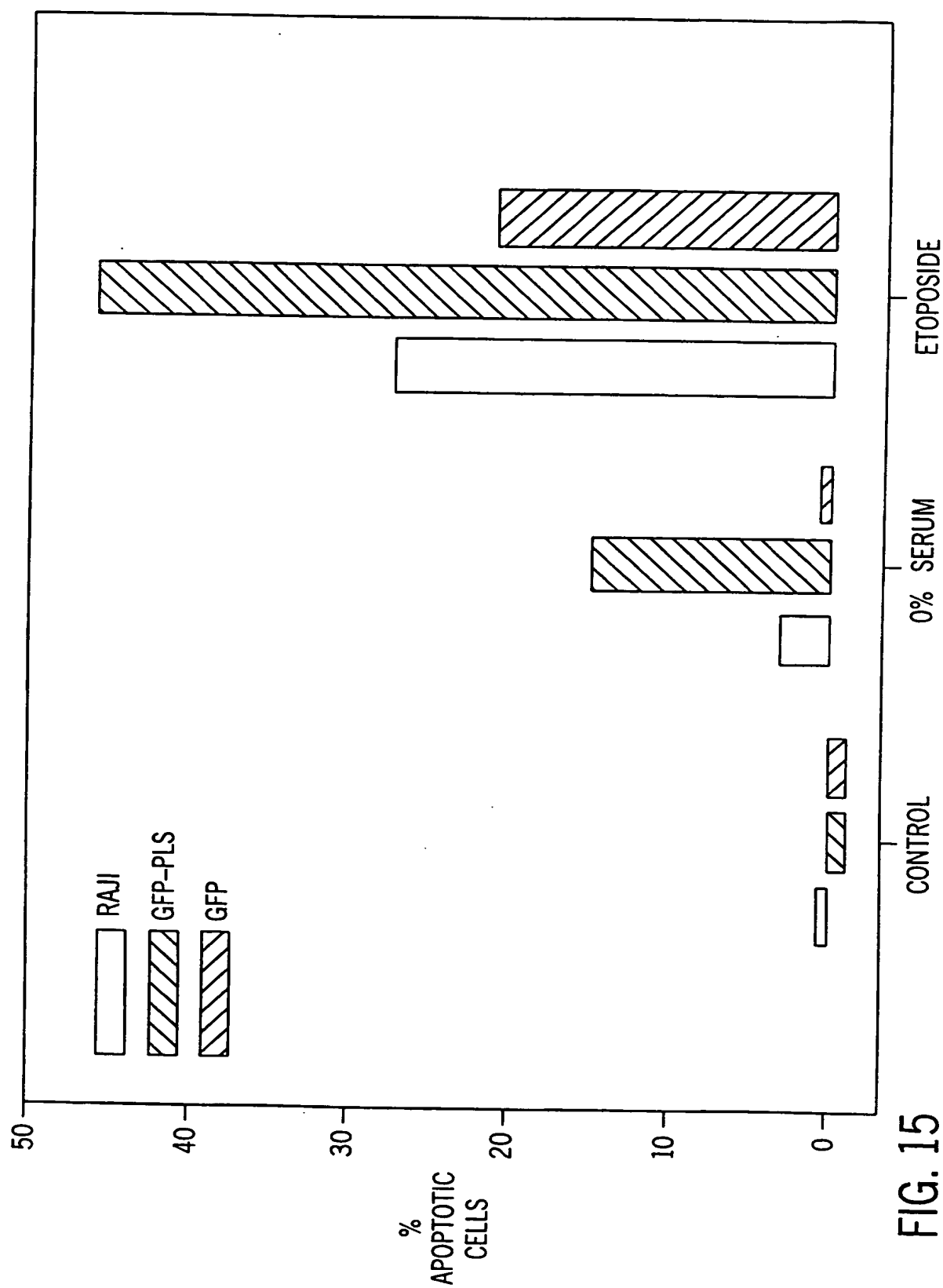


FIG. 15

## SEQUENCE LISTING

<110> Sims, Peter J.  
Zhao, Ji  
Wiedmer, Therese

<120> METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY  
TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO  
CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM

<130> 160180.90121

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 15/12, C07K 14/47, C12N 15/11, A61K 38/17, 31/70, C12Q 1/68, G01N 33/68, A61K 48/00		A3	(11) International Publication Number: <b>WO 99/36536</b> (43) International Publication Date: 22 July 1999 (22.07.99)																																								
(21) International Application Number: PCT/US99/01087 (22) International Filing Date: 19 January 1999 (19.01.99) (30) Priority Data: 60/071,950 20 January 1998 (20.01.98) US (71) Applicant (for all designated States except US): BLOOD CEN- TER RESEARCH FOUNDATION [US/US]; 638 North 18th Street, P.O. Box 2178, Milwaukee, WI 53233 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SIMS, Peter, J. [US/US]; 13386 Caminito Mar Villa, Del Mar, CA 92014 (US). WIEDMER, Therese [US/US]; 13386 Caminito Mar Villa, Del Mar, CA 92014 (US). ZHAO, Ji [US/US]; Apartment 105, 309 North Solano Hills Drive, Solano, CA 92075 (US). (74) Agent: BAKER, Jean, C.; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202-4497 (US).		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report. (88) Date of publication of the international search report: 9 December 1999 (09.12.99)																																									
(54) Title: METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM																																											
(57) Abstract																																											
<p>A method for extending the viability of mammalian cells or tissues comprising the step of inhibiting the expression of native PL scramblase within the cell or tissue is disclosed. In another embodiment of the invention, a method of decreasing the viability, metastatic or invasive potential of cancer cells, cancerous tissue, or viral-infected cell by causing increased expression or activity of PL scramblase protein within the cell or tissue is disclosed.</p>																																											
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/01087

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/47 C12N15/11 A61K38/17 A61K31/70  
C12Q1/68 G01N33/68 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 37225 A (BLOOD CENTER RES FOUND INC) 9 October 1997 (1997-10-09) cited in the application page 15, line 23 -page 16, line 19 claims 12-30,32,35-41	1-7
Y	page 20, line 10 -page 22, line 31	1-4,9, 23-26
Y	ZHOU, Q. ET AL.: "Sequences of human and mouse plasma membrane PL scramblase reveal residues required for Ca <sup>2+</sup> -induced reorganization of membrane phospholipids." MOLECULAR BIOLOGY OF THE CELL, (NOV., 1997) VOL. 8, NO. SUPPL., PAGE 292A; ABSTRACT 1693., XP002107580 abstract	1-4,9

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

22 September 1999

Date of mailing of the international search report

07. 10. 99

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Fax: (+31-70) 340-3016

Authorized officer

Andres, S

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International Application No

PCT/US 99/01087

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	B VERHOEVEN ET AL: "Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 182, no. 182, 1995, pages 1597-1601, XP002099796 ISSN: 0022-1007 the whole document ---	23-26
A	ZWAAL, R. & SCHROIT, A.: "Pathophysiologic implications of membrane phospholipid asymmetry in blood cells" BLOOD, vol. 89, 15 February 1997 (1997-02-15), pages 1121-1132, XP002107582 ISSN: 0006-4971 page 1123 page 1126, left-hand column, last paragraph -page 1127 ---	1,12-28
A	UTSUGI ET AL: "Elevated Expression of Phosphatidylseine in the Outer Membrane Leaflet of Human Tumor Cells and Recognition by Activated Human Blood Monocytes" CANCER RESEARCH, vol. 51, no. 51, 1 June 1991 (1991-06-01), pages 3062-3066 , XP002101466 the whole document ---	12-22
A	ZHOU Q ET AL: "Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids." JOURNAL OF BIOLOGICAL CHEMISTRY, (1997 JUL 18) 272 (29) 18240-4., XP002098868 cited in the application the whole document ---	19-22
A	BRATTON, D.: "Polyamine inhibition of transbilayer movement of plasma membrane phospholipids in the erythrocyte ghost" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 269, 9 September 1994 (1994-09-09), pages 22517-22523, XP002107581 ISSN: 0021-9258 the whole document ---	1-4
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## INTERNATIONAL SEARCH REPORT

International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	E F SMEETS ET AL: "Calcium-induced transbilayer scrambling of fluorescent phospholipids analogs in platelets and erythrocytes" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1195, no. 1195, 1994, pages 281-286, XP002099795 ISSN: 0006-3002 ---	
P,X	ZHOU Q ET AL: "Identity of a conserved motif in phospholipid scramblase that is required for Ca <sup>2+</sup> -accelerated transbilayer movement of membrane phospholipids." BIOCHEMISTRY, (1998 FEB 24) 37 (8) 2356-60., XP002098910 cited in the application the whole document ---	1-4,6,7, 9
P,X	ZHAO, J. ET AL.: "Level of expression of phospholipid scramblase regulates induced movement of phosphatidylserine to the cell surface" JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 MAR 20) 273 (12) 6603-6., XP002115839 the whole document ---	12,13, 16,17, 23,25
P,X	ZHAO J ET AL: "Palmitoylation of phospholipid scramblase is required for normal function in promoting Ca <sup>2+</sup> -activated transbilayer movement of membrane phospholipids." BIOCHEMISTRY, (1998 MAY 5) 37 (18) 6361-6., XP002107583 cited in the application the whole document ---	1-4,6, 10,11
P,X	STOUT J G ET AL: "Change in conformation of plasma membrane phospholipid scramblase induced by occupancy of its Ca <sup>2+</sup> binding site." BIOCHEMISTRY, (1998 OCT 20) 37 (42) 14860-6., XP002107584 cited in the application the whole document ---	1-4,6,7, 9
E	WO 99 19352 A (BLOOD CENTER RES FOUND INC) 22 April 1999 (1999-04-22) page 16, line 16 -page 24, line 5 page 29, line 25 -page 30, line 5 examples 2,4 -----	1-7,9-11

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 01087

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1,4-16,18,23-26 (as far as in vivo methods are concerned) and claims 3-17 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-11

1.1. Claims: 1-4, 6 (partially) and claim 5

A method for extending the viability of mammalian cells by inhibiting PL scramblase with an antisense RNA.

1.2. Claims: 1-4,6-7 (all partially) and claim 9

A method for extending the viability of mammalian cells by inhibiting PL scramblase with a mutant PL scramblase.

1.3. Claims: 1-4,6-7 (all partially) and claim 8

A method for extending the viability of mammalian cells by inhibiting PL scramblase with a truncated PL scramblase.

1.4. Claims: 1-4,6 (all partially) and claims 10 and 11

A method for extending the viability of mammalian cells by inhibiting or removing fatty-acid acylation of PL scramblase.

2. Claims: 12-18 and 23-26

A method for decreasing the viability, metastatic or invasive potential of cells, or for inducing apoptosis.

3. Claims: 19-22 and 27-28

A method for determining the status of a patient's cancer.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/01087

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9737225 A	09-10-1997	AU 2597797 A EP 0943096 A	22-10-1997 22-09-1999
WO 9919352 A	22-04-1999	AU 2701999 A	03-05-1999